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ning of each regular issue of the PCT Gazette.(54) Title: NOVEL VACCINE COMPOSITIONS AND METHODS OF VACCINE PREPARATION FOR VETERINARY AND
HUMAN DISEASES

(57) Abstract: The anti-pathogen vaccine of the present invention is produced in recombinant bacteria and/or transgenic plants and then administered through standard vaccine introduction method or through standard vaccine introduction method or through the oral administration. A DNA sequence encoding for the expression of an antigen of a pathogen is isolated and ligated to a promoter which can regulate the production of the surface antigen in a bacterial or transgenic plant. Preferably, a foreign gene is expressed in a portion of the plant or bacteria, and all or part of the antigen expressing plant or bacteria used for vaccine administration. In a preferred procedure, the vaccine is administered through the consumption of the edible plant as food, or the bacteria administered orally.



WO 03/013598 A2

NOVEL VACCINE COMPOSITIONS AND METHODS OF VACCINE PREPARATION FOR VETERINARY AND HUMAN DISEASES

FIELD OF THE INVENTION

This invention pertains to vaccines against animal viruses, bacteria, other pathogenic organisms and/or antigenic agents. This invention also concerns methods of preparing such vaccines. More particularly, the invention relates to edible plants expressing exogenous antigens and use of such plants as a vaccine. The invention further concerns expression of exogenous antigens in microorganisms such as bacteria, and use of such microorganisms as a vaccine. The invention further pertains to methods of preparing and administering such plant or microorganism derived vaccines.

BACKGROUND OF THE DISCLOSURE

Domestic animal diseases annually cause reductions of substantial proportions and drastic commercial impact. In developed countries where veterinary services and injected vaccines are more readily obtained, such diseases while critical are often ameliorated. This is especially true due to larger herd/flock sizes and excess food-producing capability in these countries. In the lesser developed countries, the lack of veterinary services and drugs for such diseases and the reduced food-producing capacity has a much more substantial impact on the human population, leading to food shortages and human health problems.

The usefulness of antibiotics to effectively control bacterial pathogens is becoming increasingly difficult, because of the increased occurrence of antibiotic-resistant pathogens. Because of this, the voluntary reduction of antibiotic additives to animal feeds is practiced by many producers. However, since prevention of infectious diseases is more cost effective than the ultimate treatment of the disease once it has occurred, increased attention is being focused on the development of vaccines.

Vaccines are administered to animals to induce their immune systems to produce antibodies against viruses, bacteria, and other pathogenic organisms. In the economically advanced countries of the world, vaccines have brought many diseases under control. In particular, many viral diseases are now prevented due to the development of immunization programs.

But many vaccines for such diseases as rabies, foot and mouth disease, etc. are still too expensive for the lesser developed countries to provide to their large herd/flock animal populations. Lack of these preventative measures for animal populations routinely worsens the human condition by creating food shortages in these countries.

Microbial pathogens infect a host by: entering through a break in the integument induced by trauma; introduced by vector transmission; or by interacting with a mucosal surface. The majority of animal pathogens initiate disease by the last mechanism, *i.e.*, following interaction with mucosal surfaces. Bacterial and viral pathogens that act through this mechanism first make contact with the mucosal surface where they may attach and then colonize, or be taken up by specialized absorptive cells (M cells) in the epithelium that overly Peyer's patches and other lymphoid follicles. Organisms that enter the lymphoid tissues may be readily killed within the lymphoid follicles, thereby provoking a potentially protective immunological response as antigens are delivered to immune cells within the follicles. Alternatively,

pathogenic organisms capable of surviving local defense mechanisms may spread from the follicles and subsequently cause local or systemic disease (*e.g.*, *Salmonella spp.*).

Most pathogens enter on or through a mucosal surface, with exception of insect-borne pathogens or pathogens entering the body through wounds. Pathogens that enter, through mucosal surfaces include, without limitation, *Actinomyces*, *Aeromonas*, *Bacillus*, *Bacteroides*, *Bordetella*, *Brucella*, *Compylobacter*, *Capnobcyclophaga*, *Clamydia*, *Clostridium*, *Corynebacterium*, *Eikenella*, *Erysipelothriz*, *Escherichia*, *Fusobacterium*, *Hemophilus*, *Klebsiella*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Nocardia*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Selenomonas*, *Shigelia*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Bibro*, and *Yersinia*, pathogenic viral strains from the groups *Adeiiovirus*, *Coronavirus*, *Herpesvirus*, *Orthomyxovirus*, *Picornovirus*, *Poxvirus*, *Reovirus*, *Retrovirus*, and *Rotavirus*, pathogen fungi from the general *Aspercillus*, *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus* *Histoplasma* and *Phycomyces*, and pathogenic parasites in the general *Eimeria*, *Entamoeba*, and *Trichomonas*.

Mammalian hosts infected by a pathogen mount an immune response in an attempt to overcome the pathogen. The immune system consists of three branches: mucosal, humoral, and cellular. Mucosal immunity results from the production of secretory (sIgA) antibodies in secretions that bathe all mucosal surfaces including the respiratory tract, gastrointestinal tract, and the genitourinary tract and in secretions from all secretory glands. Secretory IgA antibodies prevent colonization of pathogens on the mucosal surfaces and are a first line of defense against colonization and invasion of a pathogen through the mucosal surfaces. The production of sIgA can be stimulated wither by local immunization of the secretory gland or tissue or by presentation of an antigen to either the gut-associated lymphoid tissue (GALT or Peyer's patches) or the bronchial-associated lymphoid tissue (BALT).

Membranous microfold cells, otherwise known as M cells, cover the surface of the GALT and BALT and may be associated with other secretory mucosal surfaces. M cells act to sample antigens from the luminal space adjacent to the mucosal surface and transfer such antigens to antigen-presenting cells (dendritic cells and macrophages), which in turn present the antigen to T lymphocytes (in the case of T-dependent antigens), which process the antigen for presentation to committed B cells. B cells are then stimulated to proliferate, migrate, and ultimately transformed into antibody-secreting plasma cells producing IgA against the presented antigen.

When the antigen is taken up by M cells overlying the GALT and BALT, a generalized mucosal immunity results with sIgA against the antigen being produced by all secretory tissues in the body. Because most pathogens enter through mucosal surfaces and such surfaces make up the first line of defense to infection and facilitate the body's immune response, vaccines that can be orally administered represent an important route to stimulating a generalized mucosal immune response leading to local stimulation of a secretory immune response in the oral cavity and in the gastrointestinal tract.

Secretory IgA antibodies directly inhibit the adherence of microorganisms to mucosal epithelial cells and to the teeth of the host. This inhibition may be the result of agglutination of microorganisms, reduction of hydrophobicity or negative charge, and blockage of microbial adhesions. These anti-adherence effects are amplified by other factors such as secretory glycoproteins, continuous desquamation of surface epithelium and floral competition.

Clinical experience with human peroral poliovirus vaccine and several peroral or intranasal virus vaccines applied in veterinary medicine shown that sIgA plays a decisive role in the

protective effect by the mucosal immune system against respiratory and enteric viral infections. The effect of sIgA appears to be that of inhibiting the entry of viruses into host cells rather than prevention of attachment.

Secretory IgA antibodies directed against specific virulence determinants of infecting organism play an important role in overall mucosal immunity. In many cases, it is possible to prevent the initial infection of mucosal surfaces by stimulating production of mucosal sIgA levels directed against relevant virulence determinants of an infecting organism. Secretory IgA may prevent the initial interaction of the pathogen with the mucosal surface by blocking attachment and/or colonization, neutralizing surface acting toxins, or preventing invasion of the host cells.

Parenterally administered inactivated whole-cell and whole-virus preparations are effective at eliciting protective serum IgG and delayed type hypersensitivity reactions against organisms that have a significant serum phase in their pathogenesis (*e.g.*, human and animal pathogens such as *Salmonella typhi* and *Hepatitis B*). However, parenteral vaccines are not effective at eliciting mucosal sIgA responses and are ineffective against bacteria that interact with mucosal surfaces and do not invade (*e.g.*, human and animal pathogens such as *Vibrio cholerae*).

Oral immunization can be effective for induction of specific sIgA responses if the antigens are presented to the T and B lymphocytes and accessory cells contained within the Peyer's patches where preferential IgA B-cell development is initiated. The Peyer's patches contain helper T cells (TH) that mediate B-cell isotype switching directly from IgM cells to IgA B cells then migrate to the mesenteric lymph nodes and undergo differentiation, enter the thoracic duct, then the general circulation, and subsequently seed all of the secretory tissues of the body, including the lamina propria of the gut and respiratory tract. IgA is then produced by the mature plasma cells, complexed with membrane-bound Secretory Component, and transported onto the

mucosal surface where it is available to interact with invading pathogens. The existence of this common mucosal immune system explains in part the potential of live oral vaccines and oral immunization for protection against pathogenic organisms that initiate infection by first interacting with mucosal surfaces.

Because of simplicity of oral delivery, there is great current interest in discovering new oral vaccine technology. Appropriately delivered oral immunogens can stimulate both humoral and cellular immunity and have the potential to provide cost-effective, safe vaccines for use in developing countries where large-scale parenteral immunization of herd or other commercially produced animals is not practical or extremely difficult to implement. Such vaccines may be based upon bacterial or viral vector systems expressing protective epitopes from diverse pathogens (multivalent vaccines) or may be based upon purified antigens delivered singularly or in combination with relevant antigens or other pathogens.

A number of strategies have been developed for oral immunization, including the use of attenuated mutants of bacteria (*e.g.*, *Salmonella spp.*) as carriers of heterologous antigens, encapsulation of antigens into microspheres composed of poly-DL-lactide-glycolide (PGL), protein-like polymers-proteinoids, gelatin capsules, different formulations of liposomes, adsorption onto nanoparticles, use of lipophilic immune stimulating complexes, and addition of bacterial products with known adjuvant properties.

Underlying the development of most current vaccines is the ability to grow the disease-causing agent in large quantities. At present, vaccines are usually produced from killed or live attenuated pathogens. If the pathogen is a virus, large amounts of the virus must be grown in an animal host or cultured animal cells. If a live attenuated virus is utilized, it must be clearly proven to lack virulence while retaining the ability to establish infection and induce humoral

and cellular immunity. If a killed virus is utilized, the vaccine must demonstrate the lack of capacity of surviving antigens to induce immunization. Additionally, surface antigens, the major viral particles that induce immunity, may be isolated and administered to induce immunity in lieu of utilizing live attenuated or killed viruses.

Vaccine manufacturing often employs complex technologies entailing high costs for both the development and production of the vaccine. Concentration and purification of the vaccine is required, whether it is made from cell cultures, whole bacteria, viruses, other pathogenic organisms or sub-units thereof. Even after these precautions, problems can and do arise. With killed bacterial cells, viruses or other pathogenic organisms, there is always a chance that live pathogens survive and vaccination may lead to isolated cases of the disease. Moreover, the vaccines may sometimes be contaminated with cellular material from the culture material from which it was derived. These contaminants can cause adverse reactions in the vaccine recipient animal and sometimes even death.

Direct injection of plasmid DNA has been used as a vaccine strategy, with reports of protective immunity and cytotoxic T lymphocyte (CTL) induction in mice after i.m. injection of a DNA plasmid. The use of DNA vaccines in preclinical studies has become well established, with report of protective immunity in many different independent studies. In recent studies, both antibody and CTL responses were induced in non-human primates, although 1-2 mg of DNA was immunized on multiple occasions in these studies. However, the use of very high doses of DNA is less favorable from a process economics standpoint, therefore, there is a clear need to induce effective immunity in veterinary medicine with lower and fewer doses of DNA, as well as to increase the magnitude of the immune responses obtained.

A number of strategies available that have the potential to improve the potency of DNA vaccine include vector modification to enhance antigen expression, improvements in DNA delivery, or the inclusion of adjuvants. The monophoryl lipid A has been reported could enhance both humoral and cell-mediated immune responses to DNA vaccination against human immunodeficiency virus type 1 (Shin S *et al.*, 1997). DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes could also induce systemic and mucosal immune responses. (Shin S, *et al.*, 1998). Manmohan S had developed a delivery system for DNA vaccines, the cationic microparticles. Vitamin D3 also plays an important role in the immunization of DNA vaccine.

It is also reported that bacterial DNA-sequences called immunostimulatory sequences can be a potent adjuvant. Non-methylated, palindrome DNA-sequences containing CpG-oligodinucleotides (CpG-ODN) can activate an 'innate' immune response by activating monocytes, NK cells, dendritic cells and B-cells in an antigen-independent manner (immunostimulatory DNA sequences, ISS). Methylation of the CpG-ODN reportedly abrogates the immunogenicity of the DNA vaccine. The use of large amounts of plasmid for immunization might only overcome the low transfection efficiency *in vivo*, as well as serve as an adjuvant, driving a Th1-type response.

Plant Genetic Engineering

Various methods are known in the art to accomplish the genetic transformation of plants and plant tissues so that foreign DNA is introduced into the plant's genetic material in a stable manner, *i.e.*, a manner that will allow the foreign DNA to be passed on the plant's progeny. Two such transforming procedures are *Agrobacterium*-mediated transformation and direct gene transfer.

Agrobacterium-mediated transformation utilizes *A. tumefaciens*, the etiologic agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms that results in the formation of tumors or galls in plant tissue at the site of infection. *Agrobacterium*, which normally infects the plant at wound sites, carries a large extrachromosomal element called Ti (tumor-inducing) plasmid.

Ti plasmids contain two regions required for tumor induction. One region is the T-DNA (transferred-DNA) which is the DNA sequence that is ultimately found stably transferred to plant genomic DNA. The other region is the vir (virulence) region which has been implicated in the transfer mechanism. Although the vir region is absolutely required for stable transformation, the vir DNA is not actually transferred to the infected plant. Transformation of plant cells mediated by infection with *A. tumefaciens* and subsequent transfer of the T-DNA alone have been documented. See, e.g., Bevan, M. W. *et al.*, *Int. Rev. Genet.* 16, 357 (1982).

After several years of intense research in many laboratories, the *Agrobacterium* system has been developed to permit routine transformation of a variety of plant tissues. Representative tissues transformed by this technique include, but are not limited to, tobacco, tomato, sunflower, cotton, rapeseed, potato, poplar, and soybean.

A. rhizogenes has also been used as a vector for plant transformation. That bacterium, which incites root hair formation in many dicotyledonous plant species, carries a large extrachromosomal element called a Ri (root-inducing) plasmid which functions in a manner analogous to the Ti plasmid of *A. tumefaciens*. Transformation using *A. rhizogenes* has developed analogously to that of *A. tumefaciens* and has been successfully utilized to transform plants that include but are not limited to alfalfa and poplar.

In the case of direct gene transfer, foreign genetic material is transformed into plant tissue without the use of the *Agrobacterium* plasmids. Direct transformation involves the uptake of exogenous genetic material into plant cells or protoplasts. Such uptake may be enhanced by use of chemical agents or electric fields. The exogenous material may then be integrated into the nuclear genome. The early work with direct transfer was conducted in the dicot *Nicotiana tabacum* (tobacco) where it was shown that the foreign DNA was incorporated and transmitted to progeny plants. Several monocot protoplasts have also been transformed by this procedure including maize and rice.

Liposome fusion has also been shown to be a method for transforming plant cells. Protoplasts are brought together with liposomes carrying the desired gene. As membranes merge, the foreign gene is transferred to the protoplast.

In addition, direct gene transfer can be accomplished by polyethylene glycol (PEG) mediated transformation. PEG mediated transformation has been successfully used to transform dicots such as tobacco and monocots such as *lolium multiflorum*. This method relies on chemicals to mediate the DNA uptake by protoplasts and is based on synergistic interactions between Mg^{+2} , PEG, and possibly Ca^{+2} . See, e.g., Negrutiu, R. *et al.*, *Plant Mol. Biol.* 8, 363 (1987).

Alternatively, exogenous DNA can be introduced into cells or protoplasts by microinjection. In this technique, a solution of the plasmid DNA or DNA fragment is injected directly into the cell with a finely pulled glass needle. This technique has been used to transform alfalfa.

A more recently developed procedure for direct gene transfer involves bombardment of cells by micro-projectiles carrying DNA. In this procedure, commonly called particle bombardment,

tungsten or gold particles coated with the exogenous DNA are accelerated toward the target cells. The particles penetrate the cells carrying with them the coated DNA. Microparticle acceleration has been successfully demonstrated to lead to both transient expression and stable expression in cells suspended in cultures, protoplasts, immature embryos of plants including but not limited to onion, maize, soybean, and tobacco.

Once plant cells have been transformed, there are a variety of methods for regenerating plants. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated. In recent years, it has become possible to regenerate many species of plants from callus tissue derived from plant explants. The plants that can be regenerated from callus include monocots, such as but not limited to corn, rice, barley, wheat, and rye, and dicots, such as but not limited to sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from tissue transformed with *A. tumefaciens* has been demonstrated for several species of plants. These include but are not limited to sunflower, tomato, white clover, rapeseed, cotton, tobacco, potato, maize, rice, and numerous vegetable crops.

Plant regeneration from protoplasts is occasionally a useful technique. When a plant species can be regenerated from protoplasts, then direct gene transfer procedures can be utilized, and transformation is not dependent on the use of *A. tumefaciens*. Regeneration of plants from protoplasts has been demonstrated for plants including but not limited to tobacco, potato, poplar, corn, and soybean.

The technology developed for the creation of transgenic plants has led many investigators to study the expression of genes derived from dissimilar plant species or from non-plant genomes.

In many cases, it has been desirable to characterize the expression of recombinant proteins encoded by genes derived from viruses or bacteria. The construction of chimeric genes for expression of foreign coding sequences in plants involves ligation of non-coding regulatory elements which function in plants 5' to the DNA sequence encoding the desired protein, and ligation of a polyadenylation signal which is active in plant cells 3' to the DNA sequence encoding the desired protein.

The 5' regulatory sequences which are often used in creation of chimeric genes for plant transformation may cause either nominally constitutive expression in all cells of the transgenic plant, or regulated gene expression where only specific cells or tissues show expression of the introduced genes. The CaMV 35-S promoter, which was derived from the Cauliflower Mosaic Virus that causes a plant disease, has frequently been used to drive nominally constitutive expression of foreign genes in plants. A regulatory DNA element which was found to control the tuber-specific expression of the patatin protein is an example of developmentally specific gene expression; this patatin promoter element is known to cause the tuber-specific expression of at least some foreign genes. See, *e.g.*, H. C. Wenzler., *et al.*, (1989) *Plant Mol. Biol.* 12:41-50.

Chimeric gene constructions may also include modifications of the amino acid coding sequence of the structural gene being introduced into transgenic plants. For example, it may be desirable to add or delete amino acids in the protein to be expressed to influence the cellular localization of foreign gene product in the cells of transgenic plants.

It has been shown that the inclusion of KDEL (SEQ ID NO: 1) and HDEL (SEQ ID NO:32) amino acid sequences at the carboxy-terminus of at least one protein enhanced the recognition for that protein by the plant endoplasmic reticulum retention machinery. S. Munro and H. R. B.

Pelham, *Cell* 48, 988-997 (1987); J. Denecke, *et al.*, *EMBO-J.* 11, 2345 (1992); E. M. Herman, *et al.*, *Planta* 182, 305 (1991); C. Wandelt, *et al.*, *The Plant Journal* 2, 181 (1992). However, such modifications are problematic at best because other factors such as protein conformation or protein folding in the transformed cells may interfere with the availability of this carboxy terminus signal by the plant endoplasmic reticulum retention machinery. S. M. Haugejorden, *et al.*, *J. Biol Chem.* 266, 6015 (1991).

Oral Vaccine Methodologies Using Transgenic Plants

The high cost of production and purification of synthetic peptides manufactured by chemical or fermentation based processes may prevent their broad scale use as oral vaccines. The production of immunogenic proteins in transgenic plants and the adjuvant effect of such proteins in transgenic plants offer an economical alternative.

While oral vaccines may be an effective and inexpensive procedure for inducing secretory immune responses in animals including humans, there is a need for proven techniques that yield transgenic plants or plant tissue that can, upon direct ingestion, cause a desired immune response to a given antigen without significant side effects.

Attempts to produce transgenic plants expressing bacterial antigens of *E. coli* and of *Streptococcus mutans* have been made. Curtiss and Ihnen, WO 90/0248, published Mar. 22, 1990. Transgenic plants that express the Hepatitis B surface antigen (HBsAg) have also been made. H. S. Mason, *et al.*, *Proc. Nat. Acad. Sci. USA*, 89:11745-749 (1992).

A series of patents issued to at least certain of the present inventors in the United States have been directed at producing vaccines in plants. In U.S. Patent 5,484,719 (Lam, Arntzen) issued

January 16, 1996, a plasmid vector was described comprising recombinant hepatitis B viral surface antigen protein DNA and a plant-functional promoter capable of directing the synthesis of the cloned protein in the plant. That patent also disclosed the use of the disclosed plasmid for constructing a transgenic tobacco plant cell. In U.S. Patent 5,612,487 (Lam, Arntzen) issued March 18, 1997, a transgenic tobacco plant was described comprising a recombinant hepatitis B viral surface antigen protein in which the plant was capable of synthesizing the viral protein into antigenic particles. That patent also disclosed a method for producing an antigenic composition using the transformed tobacco plant from which the antigenic particles were recovered to be used as a vaccine. In both of these disclosures, the inventors recognized the limitations imposed with using the tobacco plant as a host for the recombinant vaccine. Tobacco alkaloids and other toxic substances require substantial purification of the vaccine.

In U.S. Patent 5,914,123 (Arntzen, Lam) issued June 22, 1999, foods were disclosed comprising transgenic plant material capable of being ingested for its nutritional value, expressing a recombinant immunogen from Hepatitis virus or from Transmissible Gastroenteritis Virus.

In U.S. Patent 6,034,298 (Lam, Arntzen, Mason) issued March 07, 2000, a transgenic plant, as well as plasmids and methods for producing same, was disclosed expressing a recombinant viral antigenic protein from Transmissible Gastroenteritis virus. Also disclosed in that patent was a vaccine, as well as methods, for producing a pharmaceutical vaccine composition against Transmissible Gastroenteritis virus.

In U.S. Patent 6,136,320 (Arntzen, Lam/Prodigene) issued October 24, 2000, an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant viral immunogen expressed in a plant, wherein said immunogen is expressed in the plant at a

level such that upon oral administration of said composition to an animal, an immunogenic response is observed, and particularly said viral immunogen being an immunogenic protein from a virus selected from the group consisting of transmissible gastroenteritis virus and hepatitis virus, and more particularly a vaccine comprising a immunogen of hepatitis virus expressed in a plant, wherein said immunogen is capable of binding a glycosylated molecule on a surface of a membrane mucosal cell. Also a plant composition comprising a viral antigen which triggers production of antibodies and which is derived from a hepatitis B virus surface antigen or transmissible gastroenteritis virus spike protein, and plant material; said antigen being a product produced by the method of expressing said immunogen in a transgenic plant, said plant material being in a form chosen from the group consisting of a whole plant, plant part, or a crude plant extract. And an anti-transmissible gastroenteritis vaccine comprising the composition of claim 8 wherein said antigen is derived from transmissible gastroenteritis virus spike protein.

In U.S. Patent 6,194,560 (Atmtzen, Mason, Haq/TAMU) 02.27.01, a synthetic *E. coli* gene which encodes LT-B, wherein said gene comprises the DNA sequence optimized for plant codon usage. As noted in that patent, those studies have not yielded orally immunogenic plant material nor have they demonstrated that it is, in fact, possible to orally immunize animals with antigens produced in transgenic plants.

Animal Diseases

Hog cholera (HC), also known as classical swine fever, is a severe systemic and hemorrhagic disease in swine caused by Hog Cholera Virus (HCV). Classical swine fever or hog cholera represents an economically important disease of swine in many countries worldwide. Under natural conditions, the pig is the only animal known to be susceptible to HC. Hog cholera is a

highly contagious disease that causes degeneration in the walls of capillaries, resulting in hemorrhages and necrosis of the internal organs. In the first instance hog cholera is characterized by fever, anorexia, vomiting and diarrhea which can be followed by a chronic course of the disease characterized by infertility, abortion and weak off-springs of sows. However, nearly all pigs die within 2 weeks after the first symptoms appear.

HC can be transmitted from the infected swine to the healthy one by direct contact. The disease can also be transmitted through contact with body secretions and excrement from infected animals. Flies, birds and human can act as vectors in transmitting the virus.

While hog cholera does not cause food-borne illness in people, it causes serious economic losses to the pig industry since it can result in widespread deaths in pigs.

Traditionally, HC syndrome is an acute disease of high morbidity and mortality. From time to time, evolution of the virus has led to a higher incidence of subacute and chronic forms.

Virulent strains induce an acute disease that is characterized by persistent fevers that can raise body temperatures as high as 107°F. Other signs of the acute form include convulsions, anorexia, leukopenia, tonsillar necrosis and lack of appetite. At day 3 – 4 of post-infection, there is a generalized viremia with the virus replicating in epithelial cells, endothelial cells and cells of the mononuclear phagocyte system. Degeneration and necrosis of endothelial cells leads to vascular compromise, ischemia, and the induction of disseminated intravascular coagulation. These vascular changes result in petechial hemorrhage of the kidneys, urinary bladder and gastric mucosa, splenic infarction and lymph node hemorrhage. Death usually occurs within 5 to 14 days following the onset on illness.

The chronic form of hog cholera causes similar clinical signs in affected swine, but there is less hemorrhage associated. Discoloration of the abdominal skin and red splotches around the ears and extremities often occur. Pigs with chronic hog cholera can live for more than 100 days after the onset of the infection.

The mild or clinically unapparent form of hog cholera seldom results in noticeable clinical signs. Affected pigs suffer short periods of illness often followed by periods of recovery. The mild strain may cause small litter size, stillbirths and other reproductive failure. High mortality during weaning may also indicate the presence of this mild strain of hog cholera.

When pregnant sows are infected with strains of lesser virulence, transplacental infection may occur. Depending on the stage of gestation, congenital infection can result in abortion, fetal mummification, stillborn and embryonic malformations. The most frequent outcome with low virulent strains is the birth of persistently infected piglets in a state of immunological tolerance and shed large quantities of virus.

Hog Cholera Virus (HCV) is a member of the *Pestivirus* genus of the *Flaviviridae* family (Francki, R.I.B. *et al.*, 1991, *Arch of Virol Supp* 2:223-233; Horzinek, M., 1991, *Arch of Virol Supp* 3:1-5; Collett, M.S., 1992, *Comparative Immunology, Microbiology and Infectious Diseases* 15: 145-154). It replicates principally in lymphocytes and vascular endothelium. Replication of most of the HC strains is restricted to the cytoplasm of the cell and does not result in cytopathic effect.

Hog cholera virus has been shown to be structurally and serologically related to bovine viral diarrhea virus (BVDV) of cattle and to border disease virus (BDV) of sheep, which also belongs to the genus *pestivirus* within the family *togaviridae*. HCV is a small single

positive-stranded RNA virus with a genome of approximately 12.3kb (Vanderhallen, H., *et al.*, 1999, *Arch Virol* 144: 1669-1677). Its genome contains a single long open reading frame (ORF) that is flanked by a 5' and 3' nontranslated region (NTR). Meanwhile, it lacks both 5' cap and a significant 3' poly(A) sequences, if it possesses any polyadenylation. The HCV is believed to encode 3-5 structural proteins of which two are possibly glycosylated. The number of non-structural viral proteins is not known.

Modified HCV vaccines (comprising attenuated or killed viruses) for combating hog cholera infection have been developed and are presently used. However, infection of tissue culture cells to obtain HCV material to be used in modified virus vaccines leads to low virus yields and the virions are very difficult to purify. Modified live virus vaccines always involve the risk of inoculating animals with partially attenuated pathogenic HCV which is still pathogenic and can cause disease in the inoculated animal or offspring and of contamination by other viruses in the vaccine. In addition the attenuated virus may revert to a virulent state. There are also several disadvantages using inactivated vaccines, *e.g.*, the risk of only partial inactivation of viruses, the problem that only a low level of immunity is achieved requiring additional immunizations and the problem that antigenic determinants are altered by the inactivation treatment leaving the inactivated virus less immunogenic. The usage of modified HCV vaccines is not suited for eradication programs.

Vaccines containing only the necessary and relevant HCV immunogenic material that is capable of eliciting an immune response against the pathogen do not have the disadvantages of modified vaccines. Classically derived and administered recombinant HCV vaccines have been disclosed that contain only certain immunogenic portions of HCV. See, *e.g.*, US Patent Nos. 5,935,582 (issued August 10, 1999), 5,925,360 (issued July 20, 1999), and 5,811,103 (issued September 22, 1998) to Meyers *et al.*

The cDNA sequence derived from the genomic RNA of HCV is a continuous sequence about 12,500 nucleotides in length. It contains one long open reading frame (ORF), starting with the ATG codon at position 364 to 366 and ending with a TGA codon as a translational stop codon at position 12058 to 12060. This ORF consists of 3898 codons capable of encoding 435 kDa of protein.

In vivo, during HCV replication in an infected cell, this protein is synthesized as a polyprotein precursor molecule that is subsequently processed to fragment polypeptides by (enzymatic) cleavage of the precursor molecule. These fragments form after possible post-translational modifications the structural and non-structural proteins of the virus. It is possible to derive a sequence that contains the genetic information for such a fragment with immunizing properties against HCV or immunological properties characteristic for HCV or contains the genetic information for a portion of such a fragment that still has the immunizing properties or the immunological properties characteristic for HCV.

Fragment polypeptides are located within the amino acid position about 1-249, 263-487, 488-688 or 689-1067. The 1-249 region essentially represents the core protein whereas the 263-487, 488-688 and 689-1067 regions essentially represent glycoproteins of 44/48 kD, 33 kD and 55 kD respectively.

HCV is 40-50 nm in diameter. It has a nucleocapsid of about 29 nm. There are fringelike projections of 6-8 nm on the surface of the virion. The buoyant density, depending on the gradient material and on the cells used to propagate the virus, has been reported between 1.12 g/ml and 1.17 g/ml.

HCV is stable at pH 5-10; but above and below these pH values, infectivity is rapidly destroyed. HCV is quickly made inactivate by lipid solvents, such as ether, chloroform and deoxycholate. Although its infectivity is lost in cell culture medium at 60°C after 10 minutes, the virus is still active in defibrated blood at 68°C after 30 minutes. Moreover, the virus can survives in frozen carcasses for long periods of time and it can remain infective in pork and pork product for months, so it is of great epizootiologic importance.

All HC strains discovered so far were clustered into two main groups and five subgroups when genomic elements of the 5' nontranslated region (Hofmann, M.A. *et al.*, 1994, *Arch Virol* 139: 217-229), E2 gene (Lowings, J.P. *et al.*, 1994, *J. Gen Virol* 75: 3461-3468; Lowings, P. *et al.*, 1996, *J. Gen Virol* 77:1311-1321) or the NS5B gene (Vilcek, S. *et al.*, 1996, *Virus Res* 43: 137-147) were compared.

The 5' to 3' genomic organization of HCV includes a nonstructural protein designated N, an encoded nucleocapsid protein designated C, a structural envelope associated glycoproteins (E) designated E0, E1 and E2, nonstructural proteins (NS), designated NS2, NS3, NS4A, NS4B, NS5A and NS5B.

The resulting polyprotein of about 3900 amino acids is co-and post translationally processed by viral as well as host cellular proteases to yield four structural and seven to eight nonstructural viral proteins (Thiel, H-J. *et al.*, 1996, *Fundamental Virology*, 3rd ed. Raven Press New York, 1059-1079).

Nucleocapsid protein C and the three envelope-associated glycoprotein E0 (gp44/48), E1 (gp33) and E2 (gp55) are the structural components of HCV. They are located within the N-terminal third of the polyprotein (Stark, R., *et al.*, 1990, *Virol* 174: 286-289). The pestiviral

capsid protein is preceded by a nonstructural protein, p23, in the polyprotein. This non-structural core protein is a putative protease exhibits autoproteolytic activity (Thiel *et al.*, 1991, *J. Virol* 65: 4705-4712; Wiskerchen, M.A., *et al.*, 1991, *J. Virol* 65: 4508-4514).

E0 lacks a typical membrane anchor and is secreted in considerable amounts from the infected cells (Rumenapf, T. *et al.*, 1993, *J. Virol*, 67:3288-3294). Although this protein exhibits RNase activity, its enzymatic action for the viral lifecycle is still unknown.

E2 and to a lesser extent, E0 were found to be the targets for triggering neutralizing antibodies against the virus. On the other hand, E1 is believed to be buried in the viral envelope (Weiland, E. *et al.*, 1990, *J. Virol* 64: 3563-3569), and hardly any anti-E1 antibodies have been described.

In the virions and in the infected cells, the glycoproteins form disulfide-linked complexes, such as, E0 homodimer with a size of 100 kDa, E1-E2 heterodimer with a size of 75 kDa, and E2 homodimer with a size of 100 kDa (Thiel, H-J. *et al.*, 1991, *J. Virol* 65:4705-4712).

Infectious bronchitis (IB) is an acute, highly contagious viral respiratory disease of chickens characterized by tracheal rales, coughing and sneezing. The poultry industry in Southern China experienced severe outbreak of IB every year, particular in recent years, other viral respiratory disease also had a high incidence in pear river delta region of China, consequently, accurate and rapid genotyping is an important factor in controlling infectious bronchitis. The causative agent of IB is infectious bronchitis virus (IBV), which is classified in the *coronaviridae* family, genus *coronavirus*, with more than 20 serotypes identified in the world. Although vaccination with Mass-typed vaccine is widely used in China, outbreaks were still reported each year. IBV

usually damage respiratory tract, but strains of IBV replicate in the kidney, oviduct, intestine and glandular also had been reported in China.

The IBV encodes three major structural proteins: the nucleocapsid protein (N), the membrane glycoprotein and S protein. The S protein can be cleaved post-translationally to release the N-terminal S-1 and C-terminal S-2 protein. The N-terminal subunit (S-1) is responsible for cell attachment, determine tissue tropism and virus-neutralizing antibody induction, whereas the C-terminal subunit (S-2) anchors S-1 to the viral envelop. The N-terminal part of the S-1 protein is variable between different serotypes and between different strains of the same serotype. Keeler and Kingham had identified two hypervariable regions (HVRs) and two conserved regions in the N-terminal part of the S-1 gene in Mass sero-typed IBV. The HVRs that contain neutralization epitopes may be located in amino acid regions 56-69 and 117-137. The two conserved regions are located at 43-47 and 229-236. Previously HI and VN is often adopted in the diagnosis of IBV, but these methods had many drawbacks, such as time and labor consuming, and furthermore HI is not so reliable.

Since Jungher *et al.*, first described antigenic differences between the Mass and Conn, the extensive antigenic diversity of IBV is well recognized. Due to immune selective pressures associated with intensive IBV vaccination and other poor bio-security practice, over 20 serotypes of the virus had been reported, and additional variant serotypes continue to emerge and cause disease.

Due to farm practice, mass-typed vaccine is widely used in China, In recent years, intensive farming of poultry industry in Southern China increased the immune selective pressure of vaccination, at the same time, other source of IBV vaccine are also brought into China due to

open door policy. The recombination between field strain and vaccine strain will also cause outbreak of IBV.

Another commercially expensive disease is Infectious Bursal Disease (IBD), also known as Gumboro disease. IBD is caused by Infectious Bursal Disease Virus (IBDV). Apart from direct contact with infected birds, it is possible that rats and mosquitoes can transmit the disease.

During the 63th General Session of the Office International des Epizooties (OIE, Paris, 15 to 19 May 1995), it was estimated that IBD has considerable socio-economic importance at the international level, as the disease is present in the more than 95% of the Member Countries (Etteradossi, N. *et al.*, 1995; Paris OIE.).

Although IBDV does not infect human, it can cause severe economic loss. The economic importance of the disease can be categorized into two main aspects. First, some virus strains may cause up to 20% mortality in chicken three weeks of age or older. Second, prolonged immunosuppression of chickens infected at early age.

Chickens from 3 and 6 weeks of age are most susceptible to IBDV infection and mortality may be high. The syndrome of IBD can be clinical or subclinical. The age susceptibility is broader in the case of very virulent IBDV strains (Van den Berg TP, *et al.*, 1991. *Avian Pathol* 20:133-143; Nunoya T, *et al.*, 1992. *Avian Dis.* 36:597-609). After a short period of time (within 24 hours after infection), bursa of Fabricius, shows lesions. There is gelatinous yellowish transudate covering the serosal surface of the bursa.

At day 3 of post-infection, due to edema and hyperemia, bursa increases in size and weight. At day 4 of post-infection, the size of the bursa has usually doubled. Later, the bursa recede in size and the transudate disappears in the subsequent days. At day 8 of post-infection, the bursa usually becomes one-third of the original weight. There are also necrotic foci and petechial hemorrhages on the mucosal surface.

Due to impairment of clotting mechanism, pectoral muscle of the infected subject becomes dehydrated with darkened discoloration. Lesions and hemorrhage of other major organs, for instance, kidney, spleen, thymus and harderian gland can also be observed.

Instead of exhibiting clinical signs, prolonged immunosuppression arises in chicken at early age (usually below three weeks of age) after catching IBD. As a result, the infected subject is more susceptible to other diseases. This predisposes the birds to other diseases such as *colisepticaemia*, *coccidiosis*, infectious laryngotracheitis, infectious bronchitis and *salmonellosis* and *colibacillosis*. Moreover, lower antibody response to vaccination to other pathogen would be resulted. In addition to the dominant suppression of the humoral immunity of infected chicken, the cell-mediated immunity was also suppressed transiently.

IBDV is a member of the genus *Birnavirus* of the *Birnaviridae* family. It primarily infects lymphoid cells, especially precursor B cells. The primary target organ of the virus, the bursa of Fabricius is the most severely affected.

IBDV is a single shelled, non-enveloped virion with icosahedral symmetry composed of 32 capsomers and it is 60 nm – 70 nm in diameter. The capsid symmetry is skewed. Buoyant density of complete IBDV particle in cesium chloride gradient range from 1.31 g/ml to 1.34 g/ml.

IBDV resists treatment with ether and chloroform. It would be unaffected by pH 2. It is still viable exposing at 56°C for five hours. Moreover, the virus is unaffected by exposure to 0.5% phenol and 0.125% thimerosal at 30°C for one hour. On the other hand, it would be inactivated at pH 12. Its infectivity would be reduced considerable when exposed to 0.5% formalin for six hours. It would even be killed when incubating at 70°C for 30 minutes.

Two serotypes of IBDV were recognized and there were several strains within each serotype. Although its presence will stimulate antibodies, type II virus does not cause clinical disease. Hence, only IBD vaccines have been made from type I IBDV nowadays. Type II antibodies do not confer protection against type I infection, neither do they interfere with the response to type I vaccine.

The high mutation rate of the RNA polymerase of IBDV leads to antigenic variation (that are, variant strains) and modification in virulence in vivo (for example, the very virulent strain). These may require special vaccines for maximum protection. Cross protection studies have shown that inactivated vaccines prepared from "classical" type I virus require a high antigenic content to provide good protection against some of these variants.

Moreover, very virulent strains of IBDV have also emerged and caused serious disease in many countries over the past decade.

IBDV consists of two segments, designated segment A and segment B, of double stranded RNA shown by polyacrylamide gel electrophoresis. The larger segment A is approximately 3.4 kb containing two open reading frames (ORF). The larger ORF is monocistronic and encodes a polyprotein which would later be auto-processed into structural protein VP2

(40 kDa – 45 kDa), VP3 (30 kDa – 32 kDa) and protease VP4 (28 kDa) of IBDV (Muller & Becht, 1982 *J. Virol.* 1982 Oct;44(1):384-392; Azad, A.A. *et al.*, 1985, *Virol* 143: 35-44; Azad A.A. *et al.*, 1987, *Virol* 161: 145-152; Hudson, P.J., *et al.*, 1986, *Nucleic Acids Res* 14: 5001-5012; Kibenge FS, *et al.*, 1997. *Arch Virol* 142:2401-2419). Meanwhile, the shorter, partially VP2 overlapping ORF encodes VP5 protein (17 kDa). The smaller segment B (approximately 218 kb) encodes the 90 kDa functional protein VP1 (Muller H, Nitschke R. 1987. *Virology* 159:174-177; Speis *et al.*, 1987, *Virus Res* 8: 127-140). The genome of IBDV contains two restriction sites of VP2, namely *AccI* and *SpeI*. Overlapping occurs between VP5 and VP2.

VP1 functions as both RNA-dependent RNA polymerase and capping enzyme for in vivo replication of the virus. It presents as a free polypeptide and as a genome-linked protein (Muller H, Nitschke R. 1987. *Virology* 159:174-177; Kibenge FS, Dhama V. 1997. *Arch Virol* 142:1227-1236).

Sequences of VP2 in various strains are highly conserved, except the central *AccI*;*SpeI* restriction fragment which is designated as the hypervariable region (Bayliss *et al.*, 1990, *J. Gen Virol* 71, 1303-1312). Sequence that is important for the virulence and attenuation of the virus were also identified in this region (Yamaguchi T, *et al.*, 1996. *Virology* 223:219-223).

VP2 is the major host-protective immunogen of IBDV that contains the antigenic sites responsible for the induction of neutralizing antibodies (Azad *et al.*, 1987, *Virol* 161: 145-152) while VP3 protein is recognized by non-neutralizing antibodies.

VP2 and VP3 form the capsid of virus. VP2 is likely to be exposed on the outer surface of the capsid while VP3 is laid inside, interacting with the viral RNA.

VP4 protease is a non-structural polypeptide. It is responsible for cleavage of the polyprotein on segment A, but it is not included in the mature virion. The presence of serine-lysine catalytic dyade accounts for its proteolytic activity (Birghan *et al.*, 2000, *EMBO Journal* 4: 114-123).

VP5 is not essential for viral replication in cell culture but it has a regulatory function and could play a key role in virus release and dissemination (Mundt *et al.*, 1997, *J. Virol* 71:5647-51).

Another disease that affects livestock is porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV).

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered to be the most economically important viral disease of intensive swine farms in Europe and North America. The syndrome first began causing swine herd problems in the late 1980's in Unites States and prior to isolation of the causative agent, was often referred to as mystery swine disease. But in the time since the virus was identified in Europe (Leiystad Virus [LV]) in 1991 (Wensvoort G., *et al.*, (1991). *Veterinary Quarterly* 13, 121-130) and in United States (VR-2332) in 1992 (Benfield D.A., *et al.*, (1992). *J. Veterinary Diagnostic Investment* 4, 127-133), PRRSV has become a significant pathogen of swine herds worldwide, with new disease phenotypes continuing to emerge.

PRRS can result in losses in neonates and nursery from respiratory disease and reproductive losses in breeding stock. As a consequence, it causes dramatic financial consequences in swine industry. However, the inherent variability in clinical signs translates into highly variable

economic losses. On a herd basis, most acute outbreaks are estimated to decrease annual production 5%-20%.

Due to the difference in prevailing health status of swine, virus strain and management strategies of different farm, clinical signs and production losses vary widely among heard. Moreover, many cases are often complicated with secondary infections that increase severity of this disease (De Jong, M.F. *et al.*, 1991, *European Comm Seminar on the New Pig Disease*, 4:29-30 #4; Benfield, D.W. *et al.*, 1992, *J. Vet Diagn Invest.* 4:127-133).

There are three phases in the acute form of PRRSV infection: the initial, climax and final phase (Raymakers, J.M.L., 1991, *European Comm Serminar on PRRS 11:4-5, Brussels, #16*). In the initial phase of the disease, clinical signs such as inappetance, lethargy, depression and pyrexia can be seen in the breeding/gestating, farrowing or grow/finish area of pig farm. During this phase, respiratory disorders, such as dyspnea and polypnea, may or may not be observed in adult pigs but these syndromes are usually prominent in younger animals. It typically lasts 1 – 3 weeks.

During the climax phase, premature farrowings, increased stillborn, mummified, and weakborn pigs, and an increase in preweaning mortality can be observed. In the same time, most of the growth reduction and mortality is due to secondary infection. Increase secondary infections occur in growing pigs, especially in the nursery (De Jong, M.F. *et al.*, 1991, *European Comm Seminar on the New Pig Disease* 4:29-30 #4; Benfield, D.A., *et al.*, 1992, *Diseases of Swine 7th Ed.* Ames, IA: Iowa State University Press: 756-762). It typically lasts 8 – 12 weeks.

In the final phase, reproductive parameters return to near-normal pre-PRRS levels and there are variable respiratory diseases in nursery or grow-finish pigs. This phase may either be the prelude to chronic disease or to a return to normal pre-PRRS production level.

During chronic PRRS, a long term reduction in the number of pig born alive can be seen (Dial, D., *et al.*, 1990, *MSD Com Mtg. Denver: Livestock Conservation Institute*, 3.6) and an extended duration of reduced farrowing rates has also been observed (Benfield, D.A., *et al.*, 1992, *Diseases of Swine 7th Ed.* Ames, IA: Iowa State University Press: 756-762; Benfield, D.W., *et al.*, 1992, *J. Vet Diagn Invest.* 4: 127-133). Pigs in chronic PRRS herds have higher feed gain ratios in addition to slower growth. Meanwhile, the increased numbers of secondary infections are probably responsible for the continued rhinitis and pneumonia observed in chronically affected herds.

Although the mechanism of reproductive failure is still unknown, transplacental infection of porcine fetuses is common in late gestation. Moreover, semen can transmit PRRSV though no virus can be isolated from the testicles or accessory sex glands of mature boar (Ohlinger, V., 1992, *Pig Dis Info Centre*).

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the genus Arterivirus of Arteriviridae family (Cavanagh, D. 1997, *Arch of Virol* 142: 629-633). Alveolar macrophages are the target cells of PRRSV in vivo.

PRRSV is a spherical, enveloped virus 45 nm-70 nm in size (Benfield, D.A., *et al.*, 1992, *Diseases of Swine 7th Ed.* Ames, IA: Iowa State University Press: 756-762; Benfield, D.W., *et al.*, 1992, *J. Vet Diagn Invest.* 4: 127-133) and it contains a icosahedral nucleocapsid core of 20 nm-30 nm. The lipid bilayer that surrounds the nucleocapsid contains two major

envelope components, GP5 and M, and two minor envelope components, GP2 and GP4. Small surface projections are also apparent. The PRRSV has a buoyant density of 1.18 g/ml – 1.19 g/ml in CsCl and 1.13 g/ml – 1.14 g/ml in sucrose. Peak infectivities are greater in CsCl purified preparations than in sucrose preparations (Benfield, D.A., *et al.*, 1992, *Diseases of Swine 7th Ed.* Ames, IA: Iowa State University Press: 756-762; Benfield, D.W., *et al.*, 1992, *J. Vet Diagn Invest.* 4: 127-133).

Although the infectivity titer of the virus is stable for more than four months at 70°C, it is reduced 10 times when maintains at 56°C for 15 – 20 minutes or at 37°C for 10-24 hours (Benfield, D.A., *et al.*, 1992, *Diseases of Swine 7th Ed.* Ames, IA: Iowa State University Press: 756-762; Benfield, D.W., *et al.*, 1992, *J. Vet Diagn Invest.* 4: 127-133). Virus infectivity titers are reduced over 90% at pH levels less than 5 or greater than 7. In addition, virus replication is inactivated after treatment with chloroform or ether.

PRRSV is made up of a polyadenylated, single-stranded positive sense RNA molecule of 15.1 kb (Meulenberg, J.J.M. *et al.*, 1993, *Virol* 192: 62-72), which consists of eight open reading frames (ORFs), designated 1a, 1b, 2, 3, 4, 5, 6 & 7 (Conzelmann, K.K. *et al.*, 1993, *Virol* 193: 329-339; Meulenberg, *et al.*, 1995. *Virology* 206:155-163). In PRRSV-infected cells, the ORFs of the virus are transcribed into 3' nested set of six messenger RNAs. All six mRNAs have 3' polyA tail and a common leader sequence obtained from the 5' end of the genomic RNA.

ORF 1a and 1b comprise 75% of the genome at the 5' end and code for protein with apparent replicase and RNA polymerase activities. ORFs 2 to 6 encode viral membrane-associated proteins. In addition, polypeptides encoded by ORFs 2-5 are glycosylated and have been designated GP2 (29kD), GP3 (43kD), GP4 (31 kD) and GP5 (25kD).

GP2 is one of the minor components in the viral envelope. A portion of it is folded on itself via disulfide bonds, without forming homodimers, or hetero-multimers with other viral protein (Meulenberg, J.J.M., *et al.*, 1996, *Viol* 225: 44-51).

GP3 can provide protection for piglets against PRRSV infection in the absence of a noticeable neutralizing humoral response, as demonstrated in the North American and European strains. There are highly hydrophobic sequences at the N-and C-terminal regions of GP4. Although anti-GP5 antibodies can neutralize PRRSV infection, it is less effective than anti-GP5 antibodies (Weiland, E., *et al.*, 1999, *Vet Microbiol* 66: 171-186).

GP5 that incorporated into the viral envelope contains N-linked oligosaccharides of the high mannose and complex type. GP5 is important for the infectivity of PRRSV as specific anti-GP5 antibodies can neutralized PRRSV infection of susceptible cells (Pirzadeh, B. *et al.*, 1997, *Viol*, 78:1867-1873).

The polypeptide, designated as M protein, encoded by ORF6 (19 kD) is a non-glycosylated type III transmembrane protein. It forms disulfide-linked heterodimers with the GP5 glycoprotein. In PRRSV infected cells, disulfide-linked M protein homodimer have also been observed but these were not incorporated into the virions (Mardassi, H. *et al.*, 1996, *Viol* 221:98-112).

ORF 7 encodes a non-glycosylated polypeptide (15 kD) that forms the nucleocapsid, designated N. N is a highly basic protein predominantly present as disulfide-linked homodimer (Mardassi, H. *et al.*, 1996, *Viol* 221: 98-112; Meulenberg, *et al.*, 1995. *Virology* 206:155-163).

Vaccines against HCV, IBDV and PRRSV

The vast economic importance of HC initiated various efforts to extinguish the disease. The live attenuated vaccine strains, for example, the Chinese strain, which is the most extensively used vaccine, can effectively protect pig against the disease.

Although vaccination with attenuated live virus is safe and effective, it interferes with serodiagnosis and does not discriminate between vaccinated and infected animals. Therefore, current control to fight against large outbreaks of HC in Europe is based largely on quarantine restriction and slaughter policy. In order to avoid trade restrictions, the slaughter policy is mostly based on the destruction of infected and serologically positive (suspected) animals. The enormous cost of eradication programs stimulated the search for alternate methods to control the disease.

Due to the robustness of IBDV, hygienic measures alone are insufficient to control the disease. Hence, the currently favored practice to control IBD is to vaccinate the parent birds with an oil-emulsion vaccine just before laying in order to induce a high level of passive immunity in the offspring, which would protect them. The progeny are then vaccinated with a killed oil emulsion vaccine at seven days old to give a protection rate of around 85% to 90% (Wyeth PJ, Chettle NJ. 1990. *Vet Rec* 126:577-578). This may be followed by live vaccine in the drinking water at around 2.5, 3.5 and 4.5 weeks of age although researchers have shown that this offers no extra protection (Wyeth & Chettle, 1990 *Vet Rec.* Jun 9;126(23):577-578; Goddard, *et al.*, 1994 *Vet Rec.* Sep 17;135(12):273-274).

However, injected vaccine will be neutralized in the presence of high level of maternal antibodies if administered too early, Hence, serological monitoring is usually necessary to determine the optimal timing for vaccination (Van den Berg TP, *et al.*, 1991. *Avian Pathol* 20:133-143).

With the occurrence of variant strains (with different antigenic properties) and very virulent strains (can breakthrough even high levels of maternal antibodies), classical IBDV vaccine becomes ineffective in defeating IBD.

Current strategies for the control of PRRSV depend largely on immunization with modified-live vaccines that have inherent drawbacks. Firstly, the live vaccine has the intrinsic risk of reversion to a virulent phenotype. Secondly, it is not possible to discriminate between vaccinated and infected animals in a herd. It has been reported that a DNA vaccine against PRRSV trigger both cellular mediated immunity and humoral immunity (Kwang, J., *et al.*, 1999, *Res in Vet Sci*, 67:199-201).

However, despite these advances, there still exists a need for effective and easy to produce and administer vaccines for HCV, IBDV and PRRSV and other animal diseases.

SUMMARY OF THE INVENTION

In order to provide a clear and consistent understanding of the present invention, the following list of terms and their definitions are provided.

An animal is any vertebrate or invertebrate, including, but not limited to humans, birds and fish.

An antigen is a macromolecule that is capable of stimulating the production of antibodies upon introduction into a mammal or other animal including humans. As used in this application, antigen means an antigen per se, an antigenic determinant or the antigen, or a fusion protein containing the antigen or antigenic determinant sometimes referred to a native epitopes.

An antigenic determinant is a small chemical complex that determines the specificity of an antigen-antibody reaction. Colonization and/or virulence antigens of a pathogen contain one or more antigenic determinants.

An amino acid domain is an amino acid sequence within a protein that can be associated with a particular function or sequence homology.

A colonization or virulence antigen is an antigen on the surface of a pathogenic microorganism that is associated with the ability of the microorganism to colonize or invade its host.

Discussion and claims may refer to colonization or virulence antigens or antigenic determinants thereof. A pathogen may contain antigens of either colonization or virulence or both and one or more DNA sequences for each or both may be transferred to a vector and used to transform a plant such that it expresses the antigen or antigens.

An immunogenic agent is any antigen that is capable of causing an immune response in animals such as upon oral ingestion of plants carrying vectors that express the antigen.

A chimeric sequence or gene is a DNA sequence containing at least two heterologous parts, *i.e.*, parts derived from, or having substantial sequence homology to pre-existing DNA

sequences which are not associated in their pre-existing states. The pre-existing DNA sequences may be of natural or synthetic origin.

A coding DNA sequence is a DNA sequence from which the information for making a peptide molecule, mRNA or tRNA are transcribed. A DNA sequence may be a gene, combination of genes, or a gene fragment.

A foreign DNA is a DNA that is exogenous to or not naturally found in the microorganisms or plants to be transformed. Such foreign DNA includes viral, prokaryotic, and eukaryotic DNA, and may be naturally occurring, chemically synthesized, cDNA, mutated, or any combination of such DNAs. The foreign DNA of this invention is derived from or has substantial sequence homology to DNA of pathogenic microorganisms and viruses, or is a synthetic gene that encodes a protein that is of similar amino acid sequence to prokaryotic genes.

A fusion protein is a protein containing at least two different amino acid sequences linked in a polypeptide where the sequences were not natively expressed as a single protein. Fusion proteins are frequently the result of genetic engineering whereby DNA sequences from different genes are joined together to encode a single protein composed of amino acid sequences from the originally separate genes.

A gene is a discrete chromosomal region that codes for a discrete cellular product.

A microorganism is a member of one of the following classes: bacteria, fungi, protozoa, or viruses.

A plant tissue is any tissue of a plant in its native state or in culture. This term includes, without limitation, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures, and any group of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type to plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue. Plants suitable for transformation according to the processes of this invention included, without limitation, monocots such as corn, wheat, barley, sorghum, rye, rice, banana, and plantains, and dicots such as potato, tomato, alfalfa, soybean, beans in general, canola, apple, pears, fruits in general, and other vegetables.

A plant transformation vector is a plasmid or viral vector that is capable of transforming plant tissue such that the plant tissue contains and expresses DNA not pre-existing in the plant tissue.

A food stuff or edible plant material is any plant material that can be directly ingested by animals or humans as a nutritional source or dietary complement.

A pre-existing DNA sequence is a DNA sequence that exists prior to its use, in toto or in part, in a product of method according to this invention. While such pre-existence typically reflects a natural origin, pre-existing sequences may be of synthetic or other origin.

An immune response involves the production of antibodies, which are proteins called immunoglobulins. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the type of foreign antigen that induced them. Binding by antibody inactivates viruses and bacterial toxins (such as tetanus or botulinum toxin) frequently by blocking their ability to bind to receptors on target cells. Antibody binding also marks invading microorganisms for destruction, either by making it easier for a phagocytic cell

to ingest them or by activating a system of blood proteins, collectively called complement, which kills the invaders. Cell-mediated immune responses, the second class of immune responses, involve the production of specialized cells that react with foreign antigens on the surface of other host cells. The reacting cell can kill a virus-infected host cell that has viral proteins on its surface, thereby eliminating the infected cell before the virus has replicated. In other cases the reacting cell secretes chemical signals that activate macrophages to destroy invading microorganisms.

A secretory immune response (SIR) is a specific type of immune response. It involves the formation and production of secretory IgA antibodies in secretions that bathe the mucosal surfaces of human and other animals and in secretions from secretory glands. An agent that causes the formation and production of such antibodies is considered to stimulate secretory immunity or to elicit a SIR. Secretory immunity is also sometimes referred to as mucosal immunity.

A substantial sequence homology is a functional and/or structural equivalence between sequences of nucleotides or amino acids. Functional and/or structural differences between sequences having substantial sequence homology is frequently de minimus

A transgenic plant is a plant that contains and expresses DNA that was not pre-existing in the plant prior to the introduction of the DNA into the plant.

Transgenic plant material is any plant matter, including, but not limited to cells, protoplasts, tissues, leaves, stems, fruit and tubers both natural and processed, containing and expressing DNA that was not pre-existing in the plant prior to the introduction of the DNA into the plant. Further, plant material includes processed derivatives thereof including, but not limited to food

products, food stuffs, food supplements, extracts, concentrates, pills, lozengens, chewable compositions, powders, formulas, syrups, candies, wafers, capsules and tablets.

An edible plant material includes a plant or any material obtained from a plant that is suitable for ingestion by mammal or other animals including humans. This term is intended to include raw plant material that may be fed directly to animals or any processed plant material that is fed to animals, including humans. Materials obtained from a plant are intended to include any component of a plant that is eventually ingested by a human or other animal.

The invention provides an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a plant, wherein the immunogen is expressed in the plant at a level such that upon oral administration of the composition to an animal, an immunogenic response is observed. In certain embodiments, the HCV, IBDV, IBV, ILTV or PRRSV immunogen is capable of generating an immunogenic response to HCV, IBDV, IBV, ILTV or PRRSV when the immunogen interacts with a mucosal membrane. In particular aspects, the HCV, IBDV, IBV, ILTV or PRRSV immunogen is capable of binding a glycosylated molecule on the surface of a membrane of a mucosal cell. In further aspects, the HCV, IBDV, IBV, ILTV or PRRSV immunogen is a chimeric protein.

A product orally acceptable immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a plant.

A product an immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a bacteria.

A product an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a plant for use as a medicament.

A product an immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a bacteria for use as a medicament.

Use of an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a plant for the manufacture of a medicament for the treatment of a disease caused by HCV, IBDV, IBV, ILTV or PRRSV.

Use of an immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a bacteria for the manufacture of a medicament for the treatment of a disease caused by HCV, IBDV, IBV, ILTV or PRRSV.

The invention also provides an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein the immunogen is expressed in the plant at a level such that upon oral administration of the composition to an animal, an immunogenic response is observed, the viral immunogen being an immunogenic protein from a cholera virus of swine.

The invention provides orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein the immunogen is

expressed in the plant at a level such that upon oral administration of the composition to an animal, an immunogenic response is observed, the viral immunogen being an immunogenic protein from a porcine reproductive and respiratory syndrome virus.

The invention provides an orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein the immunogen is expressed in the plant at a level such that upon oral administration of the composition to an animal, an immunogenic response is observed, the viral immunogen being an immunogenic protein from an infectious bursal disease virus.

The invention further provides vaccine comprising an immunogen of hog cholera virus, porcine reproductive and respiratory syndrome virus or infectious bursal disease virus expressed in a plant, wherein the immunogen is capable of binding a glycosylated molecule on a surface of a membrane mucosal cell. In certain embodiments, the immunogen is a hog cholera virus immunogen. In other embodiments, the immunogen is a porcine reproductive and respiratory syndrome virus immunogen. In additional embodiments, the immunogen is an infectious bursal disease virus.

The invention also provides a plant composition comprising a viral antigen which triggers production of antibodies and which is derived from a HCV, IBDV, IBV, ILTV or PRRSV antigen and plant material; the antigen being a product produced by the method of expressing the immunogen in a transgenic plant, the plant material being in a form chosen from the group consisting of a whole plant, plant part, or a crude plant extract.

The invention further provides a transgenic plant expressing a nucleotide sequence which encodes a recombinant viral antigenic protein, the recombinant protein derived from a cholera

virus of swine, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus. In certain aspects, the protein is chimeric. In other aspects the plant is *Arabidopsis*.

The invention additionally provides a vaccine composition comprising: a recombinant viral antigenic protein, the protein produced in a plant and derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or infectious bursal disease virus; and plant material, wherein the vaccine composition is capable of eliciting an immune response upon administration to an animal.

The invention further provides a food comprising transgenic plant material capable of being ingested for its nutritional value, the transgenic plant expressing a recombinant immunogen derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus. In certain embodiments, the plant is *Arabidopsis*.

The invention provides a food comprising transgenic plant material capable of being ingested for its nutritional value, the transgenic plant expressing a recombinant immunogen derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus. In certain aspects, the immunogen is derived from hog cholera virus. In other aspects, the immunogen is derived from a porcine reproductive and respiratory syndrome virus. In certain embodiments, the immunogen is derived from hog cholera virus. In other embodiments, the plant is selected from the group consisting of: tomato and potato. In additional aspects, the transgenic plant material is selected from the group consisting of: edible fruit, leaves, juices, roots, and seed of the plant.

The invention further provides a method for constructing a transgenic plant cell comprising: constructing a DNA vector by operably linking a DNA sequence encoding a recombinant viral antigenic protein, the recombinant protein derived from a hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus to a plant-functional promoter capable of directing the expression of the DNA sequence in the plant; and transforming a plant cell with the DNA vector. In certain embodiments, the transforming comprises *Arabidopsis* mediated transformation.

The invention additionally provides a method for producing a pharmaceutical vaccine composition, wherein the pharmaceutical vaccine composition consists of a recombinant viral antigenic protein, comprising the steps of: constructing a DNA vector by operably linking a DNA sequence encoding the recombinant viral antigenic protein, the recombinant protein derived from a swine virus which is the causative agent of cholera, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus, to a plant-functional promoter capable of directing the expression of the DNA sequence in a plant; transforming a plant with the DNA vector; and recovering the pharmaceutical vaccine composition expressed in the plant. In certain aspects, the plant is an *Arabidopsis* plant.

A method for manufacturing a product pharmaceutical vaccine composition, wherein the pharmaceutical vaccine composition consists of a recombinant viral antigenic protein, comprising the steps of: constructing a DNA vector by operably linking a DNA sequence encoding the recombinant viral antigenic protein, the recombinant protein derived from a swine virus which is the causative agent of cholera, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus, to a plant-functional promoter capable of directing the expression of the DNA sequence in a plant; transforming a plant with the DNA vector; and recovering the pharmaceutical vaccine composition expressed in the plant.

The invention additionally provides an immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a bacteria, wherein the immunogen is expressed in the bacteria at a level such that upon administration of the composition to an animal, an immunogenic response is observed. In certain aspects, the HCV, IBDV, IBV, ILTV or PRRSV immunogen is unpurified from the bacteria. In other aspects the HCV, IBDV, IBV, ILTV or PRRSV immunogen is partly purified from the bacteria. In some embodiments, the HCV, IBDV, IBV, ILTV or PRRSV immunogen is a chimeric protein. In other embodiments, the administration comprises injection. In further aspects, the administration comprises oral ingestion.

The invention also provides a vaccine comprising an immunogen of hog cholera virus, porcine reproductive and respiratory syndrome virus or infectious bursal disease virus expressed in a bacteria. In certain embodiments, the immunogen is a hog cholera virus immunogen. In other aspects, the immunogen is a porcine reproductive and respiratory syndrome virus immunogen. In additional aspects, the immunogen is an infectious bursal disease virus.

The invention further provides a method for producing an immunogenic response to an immunogen in an animal, comprising the steps of: expressing a recombinant immunogen in a bacteria, wherein the immunogen is expressed in the bacteria at a level such that upon administration of the bacteria to an animal, an immunogenic response to the immunogen is observed. In certain aspects, the immunogen is derived from HCV, IBDV, IBV, ILTV or PRRSV. In other aspects, the immunogen is partly-purified from the bacteria.

The foregoing has outlined rather broadly the features and advantages of the present invention in order that the detailed description of the invention that follows may be better understood.

Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides novel DNA vaccines and edible vaccines for veterinary infectious diseases. Traditionally, vaccines comprise attenuated and/or killed virus, sub-unit vaccine (protein based), and/or DNA vaccine (DNA based) compositions. In preferred embodiments, the present invention discloses DNA and sub-unit vaccine compositions, methods of preparation and administration. In another embodiment, disclosed herein are plant-based (*e.g.*, edible) vaccine compositions, methods of preparation and administration, including oral vaccination.

Specifically disclosed herein are DNA and plant based vaccines and methods of preparing and administering such vaccines that combine efficacy, safety, and the opportunity for serological discrimination between vaccinated and infected animals. Moreover, the vaccines and methods of using vaccines disclosed herein can elicit neutralizing antibodies that are regarded as an important specific defense against diseases including, but not limited to, HC, IBDV and/or PRRSV. The DNA vaccine disclosed herein further provides the advantages of chemical stability, as well as the ability to elicit both humoral and cell-mediate immunity. It is contemplated that certain embodiments of the present vaccine invention may provide better protection value than other vaccines against the same or like diseases. It has been demonstrated that using pcDNA3.1 inserted with VP5 – 5.2 and VP2 – 3.4 of IBDV HK46 was effective in fighting against IBDV.

As would be known to one of ordinary skill in the art, foreign antigenic agents for a vaccine may be produced using standard molecular biological techniques. For instance, in a well know

use of this type of methodology, foreign DNA for human interferon was spliced into a plasmid vector, introduced into a bacterial cell, then cloned. The gene for human interferon was be excised from a human chromosome, and a bacterial plasmid linearized, through the use of the same restriction enzyme. The interferon was gene joined with the plasmid by sticky end ligation, and the plasmid containing the interferon gene taken up by a bacterium. Each daughter bacterium inherited the interferon gene, the interferon-producing bacterial population was grown and the interferon isolated from the bacteria for administration to an animal, specifically a human patient.

Often, such methods are clinically applied to produce bacterial antigens for components of vaccines. Note that these antigens are generally located at the surface of the bacteria or are secreted molecules. Such antigens include, but are not limited to, one or more virulence molecules, secreted proteins, processed surface proteins, outer membrane proteins, capsular antigens, toxins, *pili*, and/or *flagella* antigens.

Traditional DNA vaccines or "naked DNA" requires purification to separate the recombinant plasmid from the host cells (*i.e.*, bacterial cells) to obtain the clinical grade quality of the DNA vaccine. This type of vaccine is generally administered by injection. The utility of naked DNA for mucosal vaccination may be limited by the lability of DNA in tissue fluids. Hence, it is likely that a barrier must be placed between DNA vaccines and extracellular digestive enzymes if effective mucosal delivery is to be achieved. In the presence of cationic lipids, transfection of various cell types (*e.g.*, cells of stomach and colon) with DNA vaccine was facilitated via a non-specific mechanism or a physiologic pathway present (Schmid, R.M., *et al.*, 1994, *Gastroenterol*, 32:665-670).

Disclosed herein, however, a DNA vaccine was complexed to *E. coli*. In a preferred aspect of a DNA vaccine, bacteria that is expressing a foreign immunogen expressed from a DNA vector is administered as a vaccine. Such a vaccine composition is known herein as a "DNA-Bac" vaccine. The DNA-Bac embodiment of the present invention may provide an advantage in simplicity of creation and utilization, relative to other specific types of vaccines (*e.g.*, plant vaccines, recombinant DNA vaccines, purified protein based vaccines, live or attenuated antigen vaccines, etc). Such a DNA-Bac vaccine of the present invention may provide the advantage of a reduced cost of vaccine production by lacking part or all of traditional purification processes, and thus be more cost-effective for use in the prevention or treatment of veterinary infectious diseases. Specifically demonstrated herein in a non-limiting embodiment, transformed bacterial host cells comprising a recombinant plasmid expressing an immunogen against a pig infectious virus (*i.e.*, HCV) was used as a vaccine without a further purification process. The vaccine induced both humoral and cellular immunity responses. In certain embodiments, the DNA vaccine (*e.g.*, DNA-Bac vaccine) targeted mucosal inductive sites.

In one aspect of the present invention, cultured bacteria may be prepared containing and expressing one or more genes for one or more foreign antigen(s) on one or more commercially available expression vectors, including but not limited to, plasmid, cosmid, BAC, PAC and/or P1 DNA vectors. The vector may be separated from the bacteria using methods and materials commonly utilized by those of skill in the art, including but not limited to, the use of pilot-scale plasmid preps, ultrapure 100 columns, contract CAN manufacture, large-scale plasmid preparations, EndoFree Plasmid Kits, QIAfilter Plasmid Kits, QIAGEN Plasmid Kits, Large-scale BAC/PAC/P1/cosmid preparations, QIAGEN Large-Construct Kit, High-throughput plasmid minipreps, QIAwell Plasmid Kits and the like. For example, bacteria containing the expression vector may be pelleted through centrifugation, undergo alkaline lysis and endotoxin removal steps, the vector further purified using, for example, a

commercially available QIAGEN anion-exchange chromatography apparatus, as well as isopropanol precipitation to produce ultrapure plasmid DNA. Such methods can produce, for example, up to 100 mg high-copy plasmid DNA from 20 liters of LB culture (~60 g bacterial pellet).

An example of one type of DNA vaccine used beta-Gal as reporter construct. The DNA-Bac is active both *in vitro* and *in vivo*. A DNA-Bac vaccine against the hog cholera virus induced significant enhancement in serum antibody responses and cytotoxic T lymphocyte responses as compared with naked DNA. Specifically, neutralization titers against the hog cholera virus were compared using DNA-Bac low dose, DNA-Bac high dose, a DNA vaccine and commercial attenuated vaccine, and demonstrated the effectiveness of DNA-Bac vaccine preparations at both low and high dose administrations. A rabbit fever responses assay was also conducted comparing the fever reductive ability of DNA-Bac low dose, DNA-Bac high dose, a DNA vaccine, a commercial vaccine and a control, and the efficacy of the DNA-Bac composition administered at low dose and high dose were demonstrated.

Another aspect of the present invention is a novel formulation of DNA vaccine for veterinary infection diseases. Advantages for a DNA vaccine composition, method of preparation or method of administration disclosed herein include relative low cost and flexible routes of delivery. This is of particular usefulness in the developing world. For example, Hong Kong and China veterinary vaccine market for chicken and pig farms are regionally focused, as most of the animal infectious virus are geographically localized, and vaccine manufactured by foreign firms made based on foreign strains do not give full protection most of the time.

For example, since HCV and PRRSV are two devastating viral disease of swine, they have already caused enormous financial losses worldwide. HC has already caused significant

mortality and morbidity in commercial piggeries in many countries in Europe and Asia. PRRSV is now recognized throughout North America and Europe. To protect pigs against these two diseases, every individual pig has to be subjected to two sets of vaccination schemes. Such vaccination schemes suffer from the disadvantages of being time-consuming and/or money-consuming. In order to help overcome these disadvantages, and provide maximum protection against the disease, alternative vaccination methods and compositions are disclosed herein.

Additionally, the vaccines of the present invention may be used in protection for birds. For example, infectious bursal disease (IBD) is a worldwide economically important disease to poultry industry. Infectious bursal disease virus (IBDV) has been shown to be the causative agent and it is an avian lymphotropic virus that causes immunosuppression.

Thus, in certain aspects of the present invention, a DNA-Bac vaccine is contemplated for use in animal (including but not limited to pigs and chickens), for diseases including but not limited to IBD, HC and/or PRRS. It is contemplated that in certain embodiments, the invention comprises a DNA vaccine against PRRS, and thereby having use in the pig industry in protecting pigs against that disease.

Since the site and method of delivery will affect the nature of immune response (Feltquate, D.M., *et al.*, 1997; *J. Immunol.*, 158:2278-2284; Torres, C.A.T., *et al.*, 1997, *J. Virol.*, 158:4529-4532), an appropriate procedure must be chosen that balances the practical aspects of vaccine delivery to large animals, with the desire to generate the most protective response possible. Intramuscular injection, although popular in many animal models, may be undesirable for livestock because of the potential effects on meat quality of food producing animals. Moreover, it is undesirable to have needle tracts or vaccine residues in the relevant

tissue. Delivery of plasmid into the epithelium (skin or mucosal surfaces) is considered to be the most promising site of plasmid delivery because of the immunocompetence of these tissues. These tissues have highly developed immune surveillance functions. Finally, because these tissues are the sites of entry by most pathogens, immunization at these sites is expected to be more effective in fighting against the disease.

Intramuscular injection was thought to lead to the rapid movement of DNA or DNA-transfected cells out of the injected muscle, so that the immunostimulatory events leading to antibodies production and cytotoxic T lymphocytes reactivity took place primarily in distal tissue.

Bone marrow derived antigen presenting cells, probably dendritic cells, has been demonstrated to be required for process antigen expressed from DNA plasmids (Ulmer, J.B., *et al.*, 1996, *Immunol*, 89: 59-67; Ulmer, J.B., *et al.*, 1996 *Cur Opin Immunol*, 8:531-536), though it is not clear whether the antigen presenting cells are themselves transfected with the DNA or pick up antigen from other cells.

Although muscle contains relatively few resident dendritic cells, macrophages or lymphocytes, the recent discovery at IL-15 and its high levels of expression in skeletal muscle cells (Grabstein, K.H., *et al.*, 1994, *Science*, 264:961-965) indicated that muscle cells may not be as immunologically inert as once thought. Therefore, it was also a suitable site for immunization with DNA vaccine.

Currently, DNA vaccines are delivered by intramuscular or subcutaneous injection, which can induce systematic response, but generally no mucosal immunity. In the same time, the mucosal surface of the gastrointestinal tracts is the frequent site of transmission of numerous

diseases. Hence, the mucosal immune response plays an important role in the protection against viral infection. In other embodiments, it is contemplated that a DNA vaccine of the present invention (*e.g.*, a DNA-Bac vaccine) may be orally administered.

Specifically disclosed herein are DNA vaccines, including but not limited to, pcDNA3.1-VP5-5.2 & VP2-3.4 and pHCV2.5, for use in vaccination against, but not limited to, IBDV HK45 strain and HCV Alford strain, respectively. The efficiency of these two vaccines delivered to the mucosal surface of gastrointestinal tracts was demonstrated.

There are gut-associated lymphoid tissues (GALT), which consist of the organized lymphoid tissues along the gastrointestinal tract. It includes isolated lymphoid follicles, Peyer's patches, appendix, tonsils, and mesenteric lymph nodes. In addition, many small lymphoid follicles populate the mucosa. From the mouth to the anus, these follicles are covered by specialized surface epithelial cells called M cells. They play a key role in deliver samples of foreign antigens by transepithelial transport from the lumen to organized lymphoid tissues. It is contemplated that GALT sites on mucosal surfaces may be a desirable location for contact with the vaccines of the present invention during or after administration.

Disclosed herein, the viral coat proteins were inserted into a mammalian expression vector, pcDNA3.1 and used as the DNA vaccine. The DNA vaccine, pcDNA3.1-VP5-5.2&VP2-3.4, pHCV2.5 and pcDNA3.1-ORF5 have been reported to be effective in fighting against IBD, HC and PRRS respectively when they were administered through intramuscular injection. Since intramuscular injection was undesirable for livestock because of the potential effects on meat quality of food producing animals, the effectiveness of these three DNA vaccines delivered to the mucosal surface of gastrointestinal tracts of the animals was determined.

It has been reported that combined DNA immunization could induce double-specific protective immunity and non-specific response in Rainbow Trout (Pierre *et al.*, 1998, *Virol* 249: 297-306). However, the inventors know of no report of combined DNA vaccine against pig diseases. The efficacy of vaccine compositions and methods of delivery against HCV, IBDV and/or PRRSV, along or in combination, is disclosed herein.

DNA vaccines using pHCV2.5 and pcDNA3.1-ORF5 construct have been shown to be effective in protecting pigs against HC and PRRS respectively. However, subjecting to two separated vaccination schemes was very time-consuming and money-consuming. Disclosed herein, the efficacy of combined DNA vaccine against HC and PRRS was demonstrated.

After ELISA and Western blot analysis, it was observed that IBD and HC DNA vaccines were both effective in triggering specific antibodies against the respective virus when they were delivered orally.

It could also be seen that combined HC-PRRS DNA vaccine was effective in triggering specific antibodies against HCV and PRRSV simultaneously. The antibody reaction was rather similar in quality and intensity to that obtained with separated immunization

In another aspect of the present invention, one or more selected pathogen's genes are introduced into plants and the transgenic plants induced to manufacture the encoded antigens (*e.g.*, proteins).

In a preferred aspect, the plant is edible (*e.g.*, a potato plant) and the vaccine administered by consumption. Edible vaccine embodiments possess the advantages of being inexpensive (*i.e.*, it is generally less costly to grow plants than bacteria based vaccines, and generally less

expensive to produce than other vaccine production methods that incorporate various purification steps), easy to administer (*e.g.*, the plant vaccine added to animal feed), lacking injection-related hazards and eliminating or reducing the risk of contamination of animal pathogens.

There are four generally used genetic expression transformation systems that can be used for producing transgenic plants capable of being administered as one of the active agents in oral vaccines against a desired antigen. The present invention takes advantage of all four of these expression systems of the construction of edible vaccines. It should be recognized that this list is not meant to exhaust other possible approaches. The list is simply included to provide a proper context for the scope and teaching of the present invention.

First, in transgenic plants, expression vectors including the CaMV 35S promoter and antigen coding sequences can be used to constitutively transform the plants where expression in the leaves allows for rapid analysis of gene expression and biochemical characterization of gene products.

In such plants such as but not limited to *Brassica napus* (canola), expression vectors including the 2S albumin promoter and antigen coding sequences can be used to cause seed-specific gene expression to create the production of recombinant protein in seed tissues, routinely used as animal feed, providing for the production of attractive oral immunogenicity analyses.

In plants such as but not limited to *Solanum tuberosum* (potato), expression vectors including the patatin promoter or soybean vspB promoter and antigen coding sequences can be used to cause tuber-specific gene expression to create tuber-specific production of recombinant protein

in tuber tissues routinely used as food. This provides for the production of attractive oral immunogenicity analyses.

Finally, in plants such as but not limited to *Musa acuminata* (banana), expression vectors including fruit ripening-specific promoters and antigen coding sequences can be used to transform plants that produce the recombinant protein in ripened fruit where production of recombinant protein is produced directly as candidate vaccines for ingestion studies in animals and humans.

The retention of biological properties in the recombinant proteins produced in plants, specifically ligand binding and the presentation of antigenic epitopes, is of considerable importance to the successful production of edible vaccines in transgenic plants. The ultimate test of the value of proteins of pharmacological importance is their biological activity.

Vaccines are of particular interest for studies of protein expression since their effects can be accurately quantified in animal models. In addition, relatively low amounts are required, since their effects are amplified by the immune system.

Disclosed herein, in a non-limiting example, are compositions and methods for the expression of HCV antigen(s) in transgenic plant(s) that act as an edible vaccine against Hog Cholera Virus. In a demonstration of an aspect of the present invention, local strains of a pathogen (*e.g.*, HCV) were isolated and characterized. In a specific aspect of the present invention, the E2 region was used in designing the constructs (*e.g.*, pHCV1.25 and pHCV2.5). In a particular aspect of the disclosed invention, the codon usage of HCV was changed in transgenic plants. It is contemplated that expression of one or more immunogens of one or more pathogens may likewise be enhanced by changes in codon usage when expressed in plants.

In certain aspects, it is contemplated that the vaccine inventions disclosed herein may be used alone or in combination with one or more other pharmacological or therapeutic agents. In certain aspects, such an agent may comprise a biopharmaceutical, including but not limited to, one or more cytokines including but not limited to one or more interferons, interleukins, colony stimulating factors, and/or tumor necrosis factors; one or more antisense nucleic acid compositions and the like; one or more cytokines; one or more gene therapy agents or methodologies; one or more monoclonal antibodies; one or more clotting factors; one or more additional vaccines or vaccine related compositions or methods of administration; and/or one or more hormones.

A. Construction and Generation of a DNA Vaccine

The strategy of constructing and generating a DNA IBD and HC vaccine is described herein as a merely as an exemplary aspect of the present invention. However, the present invention is not limited to the specific constructs disclosed herein. As would be understood by those of ordinary skill in the art, other constructs may be created and other genes from various pathogens may be expressed using the techniques described herein to produce a DNA vaccine. In certain preferred aspects, the DNA constructs are expressed in a microorganism (*e.g.*, a bacteria). In certain aspects, a DNA/bacterial vaccine ("DNA-Bac") is partly purified. As used herein, "partly purified" means removal of about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or greater, and any range derivable therein, of bacterial component(s) from the DNA construct and/or expressed immunogen(s). In additional aspects, a DNA vaccine is administered orally, to a mucus membrane, or a combination of both. However, it is contemplated that such a DNA and/or bacteria vaccine may be administered using any technique known to those of ordinary skill in the art, and it is further contemplated that the DNA and/or bacteria vaccine may be

administered in combination with one or more immunogenic or pharmacological agents, such as, for example, an adjuvant.

1. Design of DNA construct

For IBD vaccine, VP5-5.2 and VP2-3.4 were cloned into pcDNA (+) vector under the control of a CMV promoter.

For HC vaccine, three consecutive genes (E0, E1 and E2) that encode viral structural glycoprotein were cloned into expression vector pcDNA3.1 with CMV promoter. The recombinant plasmid was called pHCV2.5 since the size of the insert was about 2.5 kb. For PRRS vaccine, ORF5 of PRRSV that encodes the major envelope protein GP5 was cloned into expression vector pcDNA3.1 with upstream CMV promoter. This vector was designed specifically for eukaryotic expression.

These three constructs have been prepared and their identities have been confirmed previously.

2. Transformation of *E. coli* cell

50 µl thawed competent *E. coli* cell and 5 µl plasmid DNA were added to a cuvette that has been chilled on ice for 5 minutes. It was then placed into the sample chamber of a bacteria electroporator (Bio Rad *E. coli* Pulser Transformation Apparatus), which has been set to 1.8 kV. 1 ml SOC medium (Gibco BRL) was added immediately to the cuvette after applying the pulse. After 1 hour of inoculation at 37°C, 100 µl of transformed cell was spread on a LB agar plate containing ampicillin and it was incubated overnight.

3. Large-scale plasmid extraction for injection

A single transformed colony on the agar plate was picked and inoculated in 2 ml LB supplemented with ampicillin. It was grown at 37°C overnight with shaking. 1.5 ml culture was then added to 1.5 L LB supplemented with ampicillin and grown at 37°C overnight with shaking. The cells were spun down at 8000X g (Beckman JA-14 Rotor) for 10 minutes and the supernatant was discarded. The cells were resuspended in 75 ml P1 solution (50 mM glucose, 25 mM Tris, pH 8, 10 mM EDTA). 102.5 ml P2 solution (0.2 M NaOH, 1% sodium dodecyl sulfate) was added and it was allowed to stand at room temperature for 5 minutes. 150 ml P3 solution (5 M potassium acetate solution, pH 4.8) was then added. After standing on ice for 30 minutes, the mixture was centrifuged at 8000 rpm for 10 minutes (Beckman JA-14 Rotor). 375 ml isopropanol was added to the supernatant and allowed to stand at 4°C overnight. It was then centrifuged at 12000 rpm for 10 minutes (Beckman JA-14 Rotor). The pellet was resuspended in 112.5 ml water containing RNase A. 7.5 ml P3 solution and 112.5 ml 100% ethanol was then added and it was kept at -20°C for 1 hour. After centrifuging at 12000 rpm for 10 minutes (Beckman JA-14 Rotor), the pellet was washed with 150 ml 70% ethanol. It was centrifuged again at 12000 rpm for 10 minutes (Beckman JA-14 Rotor). The pellet obtained was then resuspended in 2 ml 1X PBS and its concentration measured by fluorometer.

4. Large-scale bacteria preparation for feeding

A single transformed colony containing the desired plasmid on the agar plate was inoculated in 2 ml LB supplemented with ampicillin. It was grown at 37°C overnight with shaking. 1.5 ml culture was then added to 1.5L LB supplemented with ampicillin and grown at 37°C overnight with shaking.

The cells were spun down at 8000X g for 10 minutes (Beckman JA-14 Rotor) and the supernatant was discarded. The *E. coli* cells were dried at 37°C overnight and were

resuspended in 1X PBS. It was then sonicated (Branson Sonifier 250) for 10 minutes. 1% v/v antibiotics (Penicillin-Streptomycine, Gibco BRC) were finally added to it.

B. Construction and Generation of a Plant Vaccine

The construction and generation of transgenic *A. thaliana* that expresses the HCV structural genes is described herein as a merely as an exemplary aspect of the present invention.

However, the present invention is not limited to a specific species of plant and genes derived from pathogens. As would be understood by those of ordinary skill in the art, other plants may be transformed and other genes from various pathogens may be expressed using the techniques described herein to produce a plant based, and preferably edible plant vaccine, of the present invention. In certain aspects, an immunogen expressed in a plant is partly purified. As used herein, "partly purified" means removal of about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or greater, and any range derivable therein, of plant component(s) from the DNA construct and/or expressed immunogen(s). In additional aspects, the vaccine is administered orally, to a mucus membrane, or a combination of both. However, it is contemplated that such a plant or edible vaccine may be administered using any technique known to those of ordinary skill in the art, and it is further contemplated that the plant or edible vaccine may be administered in combination with one or more immunogenic or pharmacological agents, such as, for example, an adjuvant.

1. Strategy

To construct and generating transgenic *A. thaliana* that expresses the HCV structural genes, plasmid pBI 121 was digested with *Bam*HI and *Sst*II, and the vector fragments recovered. pHCV2.5 / pHCV1.25 were also digested with *Bam*HI and *Xba*I to release the HCV2.5 and

HCV1.25 fragments for insertion into the *Bam*HI and *Xba*I digested pBS plasmid. This construct was then digested with *Bam*HI and *Sst*I. pHCV2.5 codon usage was altered by recombinant PCR and the modified HCV1.25 TA cloned into pBS. *Bam*HI and *Sst*I digestion was conducted and the fragments recovered.

The recovered fragments from above and the HCV2.5 / HCV1.25 released by *Bam*HI and *Sst*I digestion described above underwent various ligation reactions to produce the pBI 121-HCV2.5, pBI 121-HCV1.25 and pBI 121-M 1.25 vectors. These vectors were then transformed into *Agrobacterium* by electroporation. *A. thaliana* was then transformed by floral dip, and underwent kanamycin selection for transgenic plants. The production of HCV transgenic plants was confirmed with PCR, Southern hybridization and Northern hybridization. Plants producing high-level expression were screened for using Western hybridization and ELISA. Finally, animals were inoculated using oral administration of transgenic plant material. Additional details of these methods are described in the following sections.

2. Plant Material

The seeds of *Arabidopsis thaliana* Columbia was purchased from LEHLE Company, P.O. Box 2366 Round Rock, TX 78680-2366 USA. Columbia (Col-0) is wild type *Arabidopsis*. The plants grow in controlled chamber or green house at 22°C ~ 25°C with 24 hours continuous light and all the treatments for plant growth are following the standard protocol (Jose M *et al* 1998). They are briefly included, 2-days cold treatment (2°C ~ 4°C) to break dormancy of the seeds, planting the germinated seeds in 9 cm pots covered with nylon window screen, and sub-irrigating the plants with water and nutrient solution regularly. The plants begin to flower and are ready for floral dip transgene after growing 3 ~ 4 weeks. The seeds will be harvested in other 3 ~ 4 weeks after the secondary floral dip.

3. *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain LBA4404 (Ooms *et al.*, *Plant Mol. Biol.* 1, 265-276, 1982) or EHA105 (Hood *et al.*, *Transgenic Research.* 2, 208-218, 1993) were used in the project. The bacteria accommodated binary plasmid pBI121 with different DNA fragments, HCV2.5 kb, HCV1.25 kb and Modified HCV1.25 kb, encoding structural viral glycoproteins were inoculated into 4 mL of LB with kanamycin and rifampin. The tubes were shaken 36-48 hours at 25°C-28°C, 250 rpm. The small culture was added into 2 L flask with 500 mL of the same medium. The large-scale culture was shaken for about 16 hours as the small culture did. Cells were harvested by centrifugation for 20 min at room temperature at 6,000 g and then resuspended in floral dip solution to a final OD₆₀₀ of approximately 0.8 prior to use.

4. Transgene and selection

The method of plant transgene used in the research is floral dip (Steven J *et al* 1998). It is briefly included the following steps (see Example 1). Clip the primary inflorescences before 3 ~ 5 days of transgene to get more flowers from secondary bolts. About 500 mL of floral dip solution with agrobacteria prepared freshly from 1 L of culture were used for 8 ~ 10 pots of plants. The plants were covered with dark plastic bags and kept in dark overnight. After 15 ~ 24 hours the treated plants were put back to chamber or green house. The plants were dip again in the next week. The seeds (T₁) were harvested and selected on 20 ~ 30ug kanamycin/mL of half MS medium. After 10 ~ 15 days of selection the plants (T₁) with 4 ~ 6 green adult leaves and with well established roots in the selection medium were taken out of the selection medium and planted into heavily moistened potting soil. The T₂ seeds were harvested independently from each individual of T₁ plants and selected again on the same kanamycin medium. The progeny of false transgenic plants (escape from the selection) can not tolerate the kanamycin exerted to the plants. The true transgenic T₁, however, will pass the

kanamycin resistance gene to the majority of the T₂ seedlings (the ratio=3/4) and these lines would be the successfully transformed plants.

5. Identification of transgenic plants

The DNA samples for PCR were prepared from a small bit of plant leaves with the following protocol, which was successfully used in rapeseed. Small pieces of leaves (about 50 mg) were ground in EP tubes frozen with liquid N₂. After adding 50 µL of extraction buffer (100 mM KCl, 50 mM Tris HCl pH 8.0, 1% PVP, 0.5% SDS, 5 mM EDTA) the tubes were boiling for 5 min. Take out the tubes and put them on ice. Centrifuge 5 min at top speed and take the supernatant for PCR. The PCR to check transgene plants were carried out in 25 µL of PCR reaction mix, in which 2 µL of prepared DNA were included. Primer pairs located in various regions of HCV 2.5 kb, HCV 1.25 kb and modified 1.25 kb (M1.25), were used to confirm the presence of HCV gene in the transgenic plants in different individuals of the possible transgenic plants.

Southern and Northern hybridization were carried out by using genomic DNA and total RNA prepared from different transgenic lines of T₂ plants, which were confirmed by both secondary selection and PCR reaction. The probes were labeled with ³²P-dATP and hybridized to the DNA and RNA membranes according to the protocols in Molecular Cloning (Fritsch., Maniatis and Sambrook., 1989. Molecular Cloning, A Laboratory Manual. (Second Edition. pp 18.47-18.75).

6. Construction of recombinant plasmids for transgene

Binary plasmid pBI121 (Clontech Company) was digested with *Bam*HI & *Sst*I and the large vector fragment was recovered from agarose gel. Three different viral DNA fragments, HCV 2.5 kb, HCV 1.25 kb and modified HCV 1.25 kb, which encoded the HCV structural

glycoprotein E^(ms)/E₁/E₂, E₂ and modified E₂, respectively, were digested with the same restriction enzymes and recovered to insert them into the vector. The recombinant plasmid was transformed into the agrobacteria with electroporation. The transformants inserted with HCV gene were used in plant transgene.

7. Construction of HCV2.5 and HCV1.25

The HCV 2.5 kb fragment inserted into pcDNA 3.1, named pHCV2.5, for expression in mammalian cells as DNA vaccine was used in the construction. The 2.5 kb DNA fragment covers the single strand RNA genome from 1118 to 3701, which encodes viral coat protein E₂, and other two structural glycoproteins E^{ms} and E₁. A large ORF encoded by ~12.5 kb single strand RNA genome of HCV is translated to a large polyprotein about 3900 amino acid, which is cleaved into individual proteins during virus assembling. Since E₂, E^{ms} and E₁ all are located in the middle of the polyprotein, the artificial start and stop codons are joined to the construct for their correct expression in plants. The construction (see the strategy above) in brief, included the following steps.

- (1) Digest pHCV2.5 plasmid DNA with *Bam*HI and *Xba*I and recover the 2.5 kb fragment, which has been added both artificial start and stop codons on the either side.
- (2) Insert the 2.5 kb DNA fragment into pBluescript SK at the same restriction enzyme sites for getting appropriate enzyme sites to clone it into plant transgene vector pBI121.
- (3) Digest pBluescript-HCV2.5 with *Bam*HI and *Sst*I, recover the 2.5 kb fragment and insert it into the same enzyme digested plant expression vector pBI121.

- (4) Transform the constructed pBI121-HCV2.5 into *Agrobacterium* LBA4404 and EHA105 after the construct was confirmed with PCR, Southern hybridization and partially sequencing.

The same procedure was used to construct the plant expression pBI121-HCV1.25. The only difference was that the HCV 1.25 kb fragment coding E2 viral coat protein, was obtained from other clone for DNA vaccine, pHCV1.25, and the pBI121-HCV1.25 was confirmed by PCR amplification of pBI121 HCV2.5, pBI121 HCV1.25 and pBI121 1.25 PCR products of pBI121 HCV2.5 in LA4404 and EHA105; pBI121 HCV1.25 in LBA4404 and EHA105; and pBI 121 M1.25 in LBA4404 and EHA105. Addition confirmation was made by separate enzyme digestion of pBI121 HCV2.5 and pBI121 with *Bam*HI, *Sst*I + *Bam*HI, and *Pst*I. Further, pBS HCV2.5 was digested with *Eco*RI+ *Bam*HI, and *Pst*I. Southern analysis of pBI121 HCV1.25 using HCV2.5 probe was also used for confirmation, as well as DNA sequencing (SEQ ID NO:3 and SEQ ID NO:4 for HCV2.5 and HCV1.25, respectively).

8. Construction of modified HCV1.25

The modification of the HCV1.25 kb fragment was carried out with the strategy of cascade recombination PCR. Total 10 primers were designed, seven of which were modified primers and the rest of three were the adapted primers. 34 codons were changed, which consisted of 8.02% of 424 total codons in HCV1.25. The modification procedure included two steps. The changes were introduced into 6 small fragments that overlapped each other by PCR with the sequence-modified primers. Then the modified small fragments were combined together by several times recombination PCR. The modified fragment was finally cloned and sequenced to confirm their changes. SEQ ID NO:5 SEQ ID NO:53 shows the unmodified sequence of HCV1.25, and SEQ ID NO:6 AND SEQ ID NO:54 show the modified HCV1.25 sequence,

designated M1.25. M1.25 has an artificial start ATG and stop TAG codons introduced on both sides of the original HCV1.25 sequence.

After modification the codon usage of M1.25 was similar with that in plants, however there was no change of any amino acid sequence in terms of the original viral sequence. The frequencies of HCV1.25 before and after the modification, and comparison with those of *A. thalianai* are displayed in Table 1.

Table 1
The codon usage of HCV1.25, M1.25 and *A. thalianai*

Species No.	Genes	PIG 60	As of PIG	scrofa dome 11 CDS's	aa of Sus s	ATH 32	Aa of ATH	Arabidopsis 33661CDS's	Aa of AT	HCV1.25	Aa of HCV	M1.25 HCV
ARG	CGA	5.8	55.3	1.2	28.4	3.6	51.2	6.4	53.5	2.2	43.3	0
	CGC	13.1		1.9		4.7		3.7		0		0
	CGG	10.9		1.7		2.5		4.8		2.2		2.3
	CGT	4.3		1.5		14		8.8		0		0
	AGA	9.8		19.9		14.8		19		20.6		21.3
	AGG	11.4		2.2		11.6		10.9		18.3		18.9
LEU	CTA	5.1	99.1	1.2	28.1	6.9	83.5	10.1	94.5	25.2	105.2	18.9
	CTC	21.7		5.6		19.9		15.7		13.7		16.5
	CTG	49.6		11.1		5.9		10		29.8		16.5
	CTT	8.5		4.4		24.4		24.2		4.5		21.3
	TTA	4		2.4		4.4		13.2		13.7		14.2
	TTG	10.2		3.4		22		21.3		18.3		18.9
SER	TCA	8	71.9	89.6	197.3	10.8	65	18.3	88.7	9.1	38.6	9.4
	TCC	21.4		30.2		12.2		10.9		9.1		7.1
	TCG	4.2		18.3		4.9		9		0		0
	TCT	11.5		39		17.4		25		2.2		2.3
	AGC	18.7		13.4		11		11.2		9.1		7.1
	AGT	8.1		6.8		8.7		14.3		9.1		9.4
THR	ACA	11.3	54.2	50	116.6	14.8	57.6	15.9	51.2	27.5	89.3	26
	ACC	25.3		32.8		19.4		10.1		32.1		33
	ACG	8.3		10.4		5		7.6		9.1		9.4
	ACT	9.3		23.4		18.4		17.6		20.6		21.3
PRO	CCA	11.7	58.7	13.1	57.5	16.6	48.5	16.1	47.8	6.8	41	11.8
	CCC	24.7		21.8		8.2		5.2		18.3		7.1
	CCG	8.8		10		7.2		8.2		9.1		11.8
	CCT	13.5		12.6		16.5		18.3		6.8		11.8

ALA	GCA	12.7	75.2	72.9	130.1	14.8	82.3	17.4	63.5	16	50.2	16.5
	GCC	34		23.7		19.7		10		9.1		7.1
	GCG	9.5		10.7		8		8.6		11.4		9.4
	GCT	19		22.8		39.8		27.5		13.7		11.8
GLY	GGA	15	78.1	86.1	178.3	34.1	81.7	23.5	64.2	16	84.7	18.9
	GGC	33.5		32.2		10.8		8.9		18.3		14.2
	GGG	18.7		46.1		7.5		10.1		32.1		16.5
	GGT	10.9		13.9		29.3		21.7		18.3		35.5
VAL	GTA	4.5	60.7	2.7	73.2	4.3	71.4	10.2	67.2	25.2	93.8	18.9
	GTC	18.5		31		19.6		12.5		20.6		18.9
	GTG	28.7		33.4		17.9		17.3		36.6		35.5
	GTT	9		6.1		29.6		27.2		11.4		18.9
CYS	AAA	20	57	8.2	16.9	18.6	56.8	31.3	63.8	20.6	55	21.3
	AAG	37		8.7		38.2		32.5		34.4		35.5
ASN	AAC	24.4	38.6	6.6	13.8	25.3	36.3	20.7	43.8	13.7	32	14.2
	AAT	14.2		7.2		11		23.1		18.3		18.9
GLN	CAA	7.8	39.3	3.2	27.6	17.4	36.9	19.7	34.7	13.7	22.8	14.2
	CAG	31.5		24.4		19.5		15		9.1		7.1
HIS	CAC	13.8	20.3	4.1	6.8	11.6	19.4	8.6	22.7	18.3	22.8	16.5
	CAT	6.5		2.7		7.8		14.1		4.5		7.1
GLU	GAA	22.5	62.5	27.5	36.5	29.8	65.1	35	67.1	25.2	57.3	26
	GAG	40		9		35.3		32.1		32.1		33.1
ASP	GAC	29.3	49	8	15.5	25.2	53.1	17.1	54.3	27.5	50.4	30.8
	GAT	19.7		7.5		27.9		37.2		22.9		21.3
CYR	TAC	20.6	30.5	5.4	9	20.7	28.7	13.5	28.7	38.9	50.3	40.2
	TAT	9.9		3.6		8		15.2		11.4		11.8
CYS	TGC	17.5	28	6.4	12.3	9.8	17.5	7.2	18.1	32.1	34.3	21.3
	TGT	10.5		5.9		7.7		10.9		2.2		14.2
HE	TTC	22.1	36	5.8	9.6	29.3	42.5	20.3	43	18.3	43.5	18.9

9. Plant transgene

A total 10 batches of plants were treated with floral dip for transgene at that point. About 80 transgenic plants expressed as kanamycin resistance on selected medium were moved into soil. And other T₁ or T₂ plants were selecting on kanamycin medium. Some T₁ plants (growing big enough) were confirmed by PCR to prove the presence of HCV gene. All the data is summarized in Table 2.

Table 2

Summary of transgenic plants

Batch No.	Gene & Bacteria	Date of floral dip (M/D/Y)	Seed	Selection (ug/mL)	Kan ^r Plants
1	2.5/105a	09/28/00; 10/05/00	0.2	50	No*
2	1.25/4404b	03/11/00; 10/11/00	0.2	50	No
	2.5/105	as above	0.2	50	No*
3	1.25/4404	12/15/00; 12/25/00	0.2	50	No
4	M1.25/105	02/03/01; 02/15/01	No**		
	M1.25/4404	as above	No		
	1.25/105	as above	No		
	1.25/4404	as above	No		
5	M1.25/4404	03/08/01; 03/15/01	3.5	20~30	1
6	1.25/4404	03/16/01; 03/23/01	4.4	20	No
7	2.5/105	03/19/01; 03/26/01	4.4	20	42
8	1.25/105	03/31/01; 04/07/01	5	20	36
9	pBI121/4404	04/04/01; 04/12/01	1.5	20	no
10	2.5/4404	as above	2	20	no

a—2-5 = pBI121 HCV2.5, 105 = EHA105

b—1.25 = pBI121 HCV1.25, 4404 = LVA4404

*The possible transgenic seeds and plants were lost either by treating with a wrong kanamycin concentration in the selection of T₂ or contaminating with fungi.

**This batch of plants were treated too long time in floral dip at first time transformation resulting serious damages of plants. In the secondary time of floral dip, unfortunately, there was a mistake of the sugar concentration and the plants died after the secondary floral dip.

From Table 2 it is shown most of the transgenic plants were transformed with the strain EHA105 and only 1 plant was transformed with LBA4404. The transformation frequency of LBA4404 was about 1/175000, or 1/80000 if the seedlings selected with 30 µg/mL of kanamycin were deducted for these seedlings may be suffered too much high concentration of kanamycin and can not grow even transformed with kanamycin resistant gene NPT II. The

strain EHA105, however, gave the average transformation frequency of 0.02%, which was similar with the frequency reported by others. Although the frequency seems to be very low it can be accepted since about 4 gram of seeds can be got easily in one transgene experiment, which in turn would represent about 40 transgenic plants.

10. Identification of transgenic plants using PCR

The kanamycin resistant plants were moved out of the selection plates and were planted into soil. When the plants grew with 8-10 leaves in chamber a small piece of leaf was taken from each plant to extract its DNA. Different pairs of primers were used to check the presence of HCV genes in the transgenic plants of HCV2.5.

The size of PCR products always changed correctly with the size predicted according to primers ZW3 and ZW5 (SEQ ID NOS:7 and 8, respectively) which were specifically designed to amplify the BAN gene from *A. thalianai* based on the *A. thalianai* BAC clone T13M11 (GenBank access number AC005882). The expected PCR product was part of the dihydroflavol 4-reductase gene, a single copy gene in *A. thalianai*, and these primers correctly produced a 780 base pair (bp) positive control amplification product. while HCV2.5 specific primers shown in SEQ ID NOS:9 and 10 correctly produced a 321 bp amplification product; genomic DNAs of three HCV2.5 and three HCV1.25 transgenic plants were amplified with the primers F01/H06 (SEQ ID NOS:11 and 12) to produce a 2.5 kb fragment that includes sequence encoding the E0, E1 and E3 peptides, and F02/H06, (SEQ ID NOS:13 and 11), which produced intact full-length HCV2.5 and HCV1.25, respectively; and transgenic plants of M1.25 and HCV1.25 were confirmed using 2 pairs of primers corresponding with different regions of M1.25 used to confirm transgenic plant M1.25, non-transgenic plant controls, and template DNA free controls were amplified with the primers producing about 1 kb fragment) demonstrating the DNA prepared in using the disclosed methods herein was good for PCR and

confirming that there indeed were the viral DNA copies in plant genome. Specific primers used to modify the E2 gene included SEQ ID NOS:14-16. Specific amplification products included a 354 bp product of SEQ ID NOS:9 and 15, a 644 bp product of SEQ ID NOS:14 and 16, and a 1060 bp product of SEQ ID NOS:14 and 12. Almost all the kanamycin resistant plants gave a positive PCR result.

EXAMPLE 1

Agrobacterium-Mediated Transformation of *A. thaliana* Using Floral-Dip Method

A. Plant culture

1. Clip the primary inflorescences when most plants have formed primary bolts (about 3-4 weeks after planting the germinated seeds in soil).
2. Dip the plants when most secondary inflorescences are 1-10 cm tall (2-4 days after clipping)
3. Cover the plants dipped in *Agrobacterium* solution with the black plastic package to maintain humidity and leave them in a low-light or dark location overnight.
4. Remove dome and return plants to the growth chamber 12 to 24 hr after inoculation.
5. Dip the inflorescences again after 6-7 days if it is needed. The inflorescences can be dipped 3 times with 6 days between each application.
6. Allow plants to grow for a further 3-5 weeks until siliques are brown and dry.
7. Harvest seeds by gentle pulling of grouped inflorescences through fingers over a piece of clean paper.
8. Store seeds in microfuge tubes and kept at 4°C under dessication.

B. *Agrobacterium* culture

1. Inoculate *Agrobacterium* (LAB4404 or EHA105) in LB (30 µg Kan + 50 µg Rif/mL) and shake overnight at 25°C-28°C with 250 rpm.
2. Transfer the overnight culture into new flasks with LB (30 µg Kan + 50 mg Rif/mL) and continue to shake 18-24 hours at the same conditions until the culture reach stationary phase.
3. Harvest the cell by centrifugation for 20 min at room temperature at 6000 g and resuspend in floral dip solution to final OD₆₀₀ of approximately 0.80. The *Agrobacterium* is ready for dipping now.

C. Floral dip inoculation

1. Put the inoculum into a beaker and make plants inverted into this suspension such that all above-ground tissue are submerged.
2. Gently agitate the plants for 3 sec-5 sec. and move the plants out of the beaker.
3. Cover and treat the plants as above (3 in plant culture).

D. Selection for transformed plants

1. Weight the transformed seeds (about 1250 seeds = 25 mg = 50 µL) and sterilize the seeds by treating them with 95% ethanol for 30-60s and 20-50% of bleach (2.625% sodium hypochlorite, final volume) containing 0.03% of Tween 20 for 5 min. Rinse the seeds 2~3 times with sterile water.
2. Suspend the sterilized seeds in approximately 10~20 mg seeds/mL of 0.1% (w/v) agarose and plate on 0.8% of agar selection plates (with 20~30 µg Kan/mL) at a

density of approximately 3000 seeds per 150 x 15 mm² plate. The selection plates contain one-half-strength MS medium (Murashige-Skoog).

3. Seal the plates and cold-treat them for 2 days.
4. Grow for 12-15 days in a controlled environment at 24°C under 23 h light 50-100 μ Einsteins m⁻² s⁻¹. (Remove excess moisture during growth by briefly opening the plates and shaking moisture off the lid.)
5. Identify transformants as kanamycin-resistant seedlings that produce green leaves and well-established roots within the selective medium.
6. Grow some of the transformants to maturity by transplanting into heavily moistened potting soil, preferably after the development 3-5 adult leaves.
7. Genomic DNA of kanamycin resistant plants is amplified with specific primers to confirm the transgeneplants.

E. Solution and medium

1. Floral dip solution (1000 mL): 5% (w/v) of sucrose (add 100 mL of 50% sucrose), 10 mM MgCl₂ (optional) (add 10 mL of 1M MgCl₂, 0.02~3% Silwet L-77 (add 200-300 μ L/L), and add H₂O to 1000 mL.
2. LB-kanamycin/rifampicin medium: LB broth with 30 μ g kanamycin and 50 μ L Rifampicin respectively.
3. Selection medium: ½ times of MS medium and 0.8% agar with 20~30 μ g of Kanmycin/mL.

EXAMPLE 2**Demonstration of DNA Constructs in Animal Models**

BALB/c mice (male) of 7 to 8 weeks of age were provided by the Laboratory Animal Unit of The University of Hong Kong. The mice were kept and fed by the animal technicians in the animal house of Department of Zoology, The University of Hong Kong.

A. Design of Animal Inoculations

35 BALB/c mice were randomly divided into 7 groups, each group consisted of 5 mice. The approach was separately replicated in twice (demonstration 1 and demonstration 2).

Details of the embodiment of the invention was shown in Table 3:

Table 3		
Details of demonstration setup		
	Group #	Treatment
IBD vaccine	Group 1	Injected with 100 µg pcDNA3.1-VP5-5.2 & VP2-3, 4 intramuscularly.
IBD vaccine	Group 2	Fed with <i>E. coli</i> cells containing 100 µg pcDNA3.1-VP5-5.2 & VP2-3.4.
HC vaccine	Group 3	Injected with 100 µg pHCV2.5 intramuscularly.
HC vaccine	Group 4	Fed with <i>E. coli</i> cells containing 100 µg pHCV2.5.
PRRS vaccine	Group 5	Injected with 100 µg pcDNA3.1-ORF5 intramuscularly.
HC-PRRS combined	Group 6	Injected with 100 µg (pHCV2.5 and

vaccine		pcDNA3.1-ORF5) intramuscularly.
Control	Control Group	Without any treatment

In Groups 1, 3, 5 and 6, the mice were injected intramuscularly at the tibialis using 27-gauge needles. DNA vaccine was injected at a single site each time. For Groups 2 and 4, the mice were fed by using feeding needle (18060-20, Fine Science Tools).

The vaccination scheme was the same for all the mice, and it was shown in the following tables.

Table 4	
Vaccination scheme of mice in demonstration 1	
Day	Treatment
0	Bleeding & 1 st vaccination
7	Bleeding
8	2 nd vaccination
15	Bleeding
18	3 rd vaccination
28	Bleeding

Table 5	
Vaccination scheme of mice in demonstration 2	
Day	Treatment
0	Bleeding & 1 st vaccination

8	Bleeding
10	2 nd vaccination
17	Bleeding
19	3 rd vaccination
28	Bleeding

B. Blood Sampling and Serum Preparation

For the first three times, mice were bled by cutting small portion of their tails (-2 mm) and 200 µl of blood was collected. For the last time, mice were bled by cardiac puncture under ether anaesthetic, and 700 µl of blood was bled using 27-gauge needle. Blood samples were allowed to clot by incubation at room temperature for 4 hours. The clotted blood was centrifuged at 5000 rpm for 10 minutes and the serum was collected.

C. Growing CEF Cells

1 ml CEF cells (Chicken Embryo Fibroblast) were thawed from the liquid nitrogen. The cells were then resuspended in 10 ml Dulbecco's Modified Minimal Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS, Gibco BRL) and 1% antibiotics (Penicillin-Streptomycine, Gibco BRC) and seeded in a T-75 flask (Falcon). The cells were incubated at 37°C with 5% CO₂ overnight.

The 100% confluent CEF cells monolayer was washed with 1X PBS twice and detached by 0.05% trypsin EDTA (Gigco BRL) for 5 minutes. Trypsin was neutralized by 10% FCS in MEM and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in DMEM with 10% FCS and 1% antibiotics (Penicillin-Streptomycine, Gibco BRC). The cells were

seeded to two T-75 flasks and if needed, one T-175 flask in the split ratio of 1 to 3 for subculturing.

D. Growing PK-15 Cells/MARC-145 Cells

1 ml PK-15 / MARC-145 cells was thawed from the liquid nitrogen. The cells were then resuspended in 10ml Minimum Essential Medium (MEM) with 10% heat-inactivated fetal calf serum (FCS, Gibco BRL) and 1% antibiotics (Penicillin-Streptomycine, Gibco BRC) and seeded to at T-75 flask (Falcon). The cells were incubated at 37°C with 5% CO₂ overnight.

The 100% confluent cells monolayer was washed with 1X PBS twice and detached by 0.05% trypsin EDTA (Gibco BRL) for 5 minutes. Trypsin was neutralized by 10% FCS in MEM and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in MEM with 10% FCS and 1% antibiotics (Penicillin-Streptomycine, Gibco BRC). The cells were seeded to two T-75 flasks and if needed, one T-175 flask in the split ratio of 1 to 3 for subculturing.

E. Virus Purification from Tissue Culture

CEF, PK-15 and MARC-145 cell were used for the amplification of IBDV, HCV and PRRSV respectively. The cell lines were infected with the respective virus in DMEM (for CEF) or MEM (for PK-15 and MARC-145) supplemented with 10% heat-inactivated FBS for 5 days. Virus was released from the cells by freezing and thawing for 3 times. Any cells attached on the culturing flasks were scraped off. Detached cells and cell debris were removed by centrifugation at 2000 rpm for 10 minutes. The supernatant containing the partially purified virus was then centrifuged at 30000 rpm for 2 hours with a Beckman 40-Ti rotor. The pellet

containing the purified virus was finally resuspended in TNE for uses in ELISA and Western blotting.

F. Immunological Techniques

IBDV, HCV, PRRSV and the prestained protein markers (broad range, Bio-Rad) were mixed with 6X loading buffer (30 mM Tris-Cl, pH 6.8, 30% glycerol, 10% SDS, 600 mM Dithiothreitol, 0.012% bromophenol blue) and denatured in boiling water bath for 10 minutes. The denatured viral proteins were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a vertical electrophoresis unit (Hoefer Scientific Instruments). The sample were first concentrated in 5% stacking gel and then resolved in 12% separating gel. Electrophoresis was performed in protein running buffer at 80 V for 2.5 hours. The gel was stained with coomassie blue or used for the Western Blot.

1. Western blotting

The resolved protein bands on the SDS-PAGE gel, the bands were transferred to absolute methanol-soaked protein membrane (Immun-Blot PVDF membrane, Bio-Rad) in the vertical unit with transfer buffer at a current of 40 mA overnight.

The membrane was then rinsed with PBS and blocked in blocking agent (5% non-fat milk and 1% Tween-20 in 1X PBS) at room temperature (RT) for 15 minutes with shaking. The blocking agent was removed and the mice anti-sera, diluted in blocking reagent, was added and incubated at RT for 2 hours with shaking. The membrane was then washed with PBS five times at 5 minutes each. After then, alkaline phosphatase (AP) conjugated goat anti-mouse IgG (Zymed, 1:50), which acted as secondary antibodies, was added to the membrane and incubated at RT for 1.5 hours. The membrane was rinsed with PBS as above. 10%

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and 10% Nitroblue tetrazolium salt (NBT) were finally added as a substrate. Colour was allowed to be developed in the dark overnight.

2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was modified to measure IBCV-, HCV-and PRRSV-specific antibody in vaccinated mice. Purified IBDV, HCV or PRRSV was firstly diluted at 1:100 using coating buffer, PBSN (15 mM Na₂CO₃, 35 mM NaHCO₃ and 0.05% NaN₃, pH 9.6 in 1X PBS). 100 µl of diluted virus was added to each well of a 96-wells ELISA plate at 4°C overnight. The plate was washed with PBST (0.05% Tween-20 IN 1X PBS) three times at 5 minutes each. The non-specific binding sites were then block by PBS with 1% Bovine Serum Albumin (BSA, USB Inc.) at 37°C for 2 hours. 100 µl diluted mouse serum (1:50) was added to the antigen-coated wells in duplication as primary antibody and incubated at 37°C for 1 hour. The plate was then washed with PBS as above. After then, HRP-conjugated goat anti-mouse IgG (Zymed, 1:5000) was added as secondary antibody and incubated at 37°C for 1 hour. The plate was then washed with PBST as above. 100 µl substrate TMB (Zymed) was added to each well and allowed to react in dark for 15 minutes. 100 µl stop solution (1N HCl) was finally added to stop the reaction. OD reading of ELISA plate was measured at 490 nm with microplate reader (Bio-Rad Model 550).

EXAMPLE 3

During the whole immunization scheme (Example 2), three of the mice died after vaccination or blood collection. This included one mouse from group 4 and one mouse from group 7 in demonstration 1 and one mouse from group 2 in demonstration 2.

In order to assess the ability of the DNA construct to elicit specific antibodies in animal model (*i.e.*, BALB/c mice), ELISA and Western blotting were performed.

A. ELISA

ELISA was performed to investigate if there was any humoral immune response in the vaccinated mice. Specific anti-IBDV, anti-HCV or anti-PRRSV antibodies in the mice sera were tested by the modified ELISE in duplicate.

The results are shown from Table 6 to Table 26 on subsequent pages. They were summarized in the following table:

Table 6				
The humoral immune response of mice from groups 3 and 4 after vaccination.				
	Demonstration 1		Demonstration 2	
	# of mice producing specific antibodies	Total number of mice	# of mice producing specific antibodies	Total number of mice
Group 1 (IBD vaccine)	1	5	3	5
Group 2 (IBD vaccine)	3	5	4	4
Group 3 (HC vaccine)	3	5	2	5
Group 4 (HC	3	4	3	5

vaccine)				
Group 5 (PRRS vaccine)	3	5	3	5
Group 6 (HC vaccine)	5	5	2	5
Group 6 (PRRS vaccine)	3	5	2	5

- Although most of the mice sera showed a slight decrease in ELISA reading in the last time when compared to that in the third time, the reading was still higher than the initial value. Hence, the DNA vaccine applied on them was proven to be effective.

Transient increase occurred when the ELISA reading of a particular mouse increased after the first and/or the second vaccination. However, the reading dropped to a level similar to that in Day 0 (pre-treatment) afterwards.

Among the 10 mice injected with pcDNA3.1-VP5-5.2 & VP2-3.4 (group 1), mouse 4 from demonstration 1 (Table 7) and mice 1, 3 and 5 from demonstration 2 (Table 8) showed an increase in IBDV-specific antibody.

Table 7					
IBDV specific ELISA reading of group 1 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
1	1	0.189	0.149	0.209	0.236
	2	0.261	0.171	0.189	0.281

	3	0.252	0.245	0.176	0.186
	4	0.137	0.248	0.358	0.590
	5	0.158	0.235	0.205	0.252

Table 8					
IBDV specific ELISA reading of group 1 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
1	1	0.416	0.416	0.574	0.654
	2	0.535	0.505	0.638	0.549
	3	0.166	0.256	0.319	0.314
	4	0.251	0.209	0.337	0.291
	5	0.224	0.335	0.379	0.368

Among the 9 mice fed with *E. coli* cells containing 100 µg pcDNA3.1-VP5-5.2 & VP2-3.4 (group 2), mice 1, 2 and 3 from demonstration 1 (Table 9) and all mice from demonstration 2 (Table 10) showed an increase of IBDV-specific antibody after vaccinating orally.

Table 9					
IBDV specific ELISA reading of group 2 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
2	1	0.420	0.626	0.654	0.638
	2	0.389	0.530	0.660	0.714
	3	0.378	0.526	0.659	0.579
	4	0.614	0.495	0.547	0.601
	5	0.638	0.593	0.628	0.600

Table 10					
IBDV specific ELISA reading of group 2 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
2	1	0.379	0.492	0.294	0.836
	2	0.434	0.640	0.569	0.885
	3	0.472	0.277	0.527	0.626
	4	0.406	0.382	0.410	0.747
	5*	0.440	0.464	/	/

*Mouse number 5 was dead after collecting blood at day 7

Among the 10 mice injected with pHCV2.5 (group 3), mice 2, 3 and 4 from demonstration 1 (Table 11) showed an increase in ELISA reading. Meanwhile, mice 4 and 5 from demonstration 2 (Table 12) showed transient increase of HCV-specific antibody.

Table 11					
HCV specific ELISA reading of group 3 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
3	1	0.837	0.740	0.916	0.792
	2	0.326	0.555	0.858	0.661
	3	0.404	0.993	1.102	0.934
	4	0.727	0.712	0.956	0.749
	5	0.684	0.846	0.758	0.518

Table 12					
HCV specific ELISA reading of group 3 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
3	1	0.772	0.621	0.484	0.579
	2	0.690	0.527	0.482	0.741
	3	0.659	0.513	0.549	0.673
	4	0.746	0.543	0.827	0.627
	5	0.406	0.598	0.480	0.529

Among the 9 mice fed with *E. coli* cells containing 100 µg pHCV2.5 (group 4), mice 1, 2 and 5 from demonstration 1 (Table 13) and mice 1, 3 and 5 from demonstration 2 (Table 14) showed an increase of HCV-specific antibody, though mouse 5 from the demonstration 2 only showed a transient increase.

Table 13					
IBDV specific ELISA reading of group 4 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
4	1	0.743	0.899	0.989	0.948
	2	0.809	0.936	0.965	1.097
	3*	/	/	/	/
	4	1.002	1.135	1.102	1.115
	5	0.785	0.853	0.991	1.009

*Mouse number 3 was dead before the start of the vaccination scheme.

Table 14					
HCV specific ELISA reading of group 4 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
5	1	0.660	0.802	0.644	0.869
	2	0.924	0.887	0.801	0.711
	3	0.523	1.088	0.910	0.790
	4	0.849	0.870	0.735	0.880
	5	0.648	0.981	0.855	0.713

Among the 10 mice injected with pcDNA3.1-ORF5 (group 5), mice 3, 4 and 5 from demonstration 1 (Table 15) and mice 1, 2 and 5 from demonstration 2 (Table 16) showed an increase in PRRSV-specific antibody level after vaccination.

Table 15					
PRRSV specific ELISA reading of group 5 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
5	1	0.513	0.424	0.390	0.419
	2	0.483	0.358	0.681	0.500
	3	0.278	0.425	0.572	0.514
	4	0.220	0.451	0.525	0.433
	5	0.396	0.473	0.749	0.551

Table 16		
PRRSV specific ELISA reading of group 5 vaccinated mice		
		ELISA reading

Group number	Code of mice	ELISA reading			
Group number	Code of mice	Day 0	Day 7	Day 15	Day 28
	2	0.300	0.331	0.347	0.466
	3	0.371	0.245	0.425	0.462
	4	0.253	0.353	0.319	0.282
	5	0.285	0.316	0.404	0.701

Among the 10 mice injected with combined vaccine of pHCV2.5 and pcDNA3.1-ORF5 (group 6), all mice from demonstration 1 (Table 17) and mice 4 and 5 from demonstration 2 (Table 18) showed an increase of HCV-specific antibody. In the meantime, mice 3, 4 and 5 from demonstration 1 (Table 19) and mice 2 and 4 from demonstration 2 (Table 20) showed an increase of PRRSV-specific antibody.

Table 17					
HCV specific ELISA reading of group 6 vaccinated mice					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
6	1	0.304	0.915	0.755	1.000
	2	0.340	0.939	0.851	0.611
	3	0.267	0.814	0.922	0.737
	4	0.197	0.601	0.764	0.688
	5	0.424	0.951	0.932	0.848

Table 18		
HCV specific ELISA reading of group 6 vaccinated mice		
		ELISA reading

Group number	Code of mice	ELISA reading			
Group number	Code of mice	Day 0	Day 7	Day 15	Day 28
	2	0.733	0.553	0.716	0.737
	3	0.897	0.453	0.834	0.831
	4	0.862	0.556	0.729	0.999
	5	0.626	0.441	0.819	1.082

Table 19

PRRSV specific ELISA reading of group 6 vaccinated mice

Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
6	1	0.380	0.420	0.395	0.380
	2	0.355	0.390	0.525	0.405
	3	0.395	0.365	0.470	0.760
	4	0.455	0.365	0.370	0.720
	5	0.510	0.350	0.365	0.685

Table 20

PRRSV specific ELISA reading of group 6 vaccinated mice

Group number		ELISA reading			
		Day 0	Day 7	Day 15	Day 28
	1	0.460	0.595	0.385	0.425
	2	0.390	0.430	0.375	0.855
	3	0.450	0.430	0.385	0.380
	4	0.415	0.440	0.565	0.400
	5	0.435	0.370	0.365	0.385

None of the mice in the negative control groups (Table 21 to Table 26) showed any increase in the anti-HCV or anti-PRRSV antibodies levels.

Table 21					
IBDV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control group*	1	0.434	0.445	0.319	0.254
	2	0.389	0.422	0.308	0.260
	3	0.284	0.320	0.239	0.222
	4	0.252	0.312	0.302	0.202

*Mouse number 5 was dead before the start of the vaccination scheme

Table 22					
IBDV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control Group	1	0.340	0.206	0.315	0.218
	2	0.417	0.417	0.316	0.284
	3	0.266	0.282	0.353	0.205
	4	0.333	0.254	0.206	0.222
	5	0.242	0.328	0.247	0.212

Table 23					
HCV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control group*	1	0.686	0.626	0.549	0.515

	2	0.582	0.593	0.604	0.568
	3	0.533	0.530	0.453	0.489
	4	0.500	0.593	0.586	0.507

*Mouse number 5 was dead before the start of the vaccination scheme

Table 24					
HCV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control Group	1	0.647	0.480	0.650	0.599
	2	0.844	0.904	0.609	0.543
	3	0.572	0.519	0.500	0.435
	4	0.772	0.710	0.782	0.560
	5	0.508	0.625	0.587	0.461

Table 25					
PRRSV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control group*	1	0.243	0.242	0.180	0.203
	2	0.152	0.235	0.203	0.230
	3	0.185	0.201	0.169	0.204
	4	0.188	0.253	0.205	0.202

*Mouse number 5 was dead before the start of the vaccination scheme

Table 26					
PRRSV specific ELISA reading of mice in the control group					
Group number	Code of mice	ELISA reading			
		Day 0	Day 7	Day 15	Day 28
Control Group	1	0.218	0.197	0.233	0.235
	2	0.278	0.358	0.196	0.159
	3	0.218	0.249	0.289	0.169
	4	0.271	0.171	0.193	0.178
	5	0.217	0.234	0.241	0.209

B. Western Blotting

Western blot analysis showed whether the viral envelope protein has been expressed.

Moreover, it also demonstrated the specificity of mice-antisera to the viral protein.

Since the viral proteins were heat-treated at 100°C for 10 minutes and resolved in the SDS-PAGE, so the viral protein were denatured. Therefore, only linear epitopes, but not conformation dependent epitopes, could be detected on the blotting membrane.

All the mice sera (collected on day 28) in a group were pooled together and diluted with blocking agent for the Western Blot analysis.

All mice sera in demonstrations 1 and 2 showed positive result in the Western blot analysis, although there was variation in intensity of the bands.

There was no banding pattern for control mice sera pooled from demonstration 1 or pooled from demonstration 2 against IBDV, HCV and PRRSV protein. In the other words, there were no specific anti-IBDV, ant-HCV or anti-PRRSV antibodies produced.

In Western blot of group 1 and 2 mice sera, wherein pooled sera from demonstration 1 and pooled sera from demonstration 2 were tested, there was a band between 36.4 kD and 48.6 kD. It corresponded to VP 2 of IBDV. The viral proteins were run in duplicate for each test.

In Western blot of group 3 mice sera, there were two bands between 48.6 kD and 96 kD. The viral proteins were run in duplicate for each test. One band was closer to 48.6 kD in the pooled mice anti-sera from demonstration 1 and represented the E2 protein of HCV (55 kD). There was an additional band between 29.8 kD and 36.4 kD. The band corresponded to E1 of HCV (33 kD). Meanwhile, one band was closer to 94 kD in the pooled mice anti-sera from demonstration 2 which corresponded to E1-E2 heterodimer of HCV (75 kD). Glycoprotein E0 has been described as a 44 kD to 48 kD protein and was observed slightly lower than 48.6 kD on the blotting membrane.

The result of Western blot of group 4 mice sera was similar to that in group 3.

In case of group 5 mice sera, there was a band just below 29.8 kD. It corresponded to GP5 of PRRSV. In the demonstration 1 test, the strong band was between 29.8 kD and 36.4 kD. Although typical GP5 was 24.5 kD – 26 kD, this strong band should represent GP5 since pcDNA3.1-ORF5 was injected. The difference may due to incomplete denaturation of the protein and resolving ability of the polyacrylamide gel.

The Western blot of group 6 mice sera showed, on the lane with HCV glycoprotein in the test of the demonstration 1 anti-sera, a band just higher than 94 kD that represented E2-E2 homodimer of HCV (100 kD). Glycoprotein E0 (44 kD to 48 kD) was observed slightly lower than 48.6 kD on the blotting membrane. Moreover, there was a band between 29.8 kD and 36.4 kD. The band corresponded to E1 of HCV (33 kD). In the test of the demonstration 2 antisera, on the lane with HCV glycoprotein, there was a band close to 94 kD. It corresponded to E1-E2 heterodimer of HCV (75 kD).

On the lane with PRRSV glycoprotein, there was a band just below 29.8 kD. It corresponded to GP5 of PRRSV in both the demonstration 1 and demonstration 2 anti-sera tests. Above the GP5 band, there were several bands. They may be the incomplete denatured GP5 or the disulfide-linked M & GP5 heterodimer.

C. Results

In the modified ELISA analysis, although ELISA plates have been blocked by blocking agent to avoid non-specific binding, non-specific background staining was still in some samples wells. For example, in group 3 mice in demonstration 1, initial reading of number 1, 4 and 5 showed a deviated value from that of number 2 and 3 which was run on another plate. Hence, the trend of ELISA reading over time has also been taken into consideration for the evaluation of specific antibodies production.

In ELISA analysis, 5 out of 10 mice injected with pcDNA3.1-VP5-5.2&VP2-3.4 have shown to produce specific antibodies against IBDV. In the same time, 7 out of 9 mice fed with *E. coli* containing pcDNA3.1-VP5-5.2&VP2-3.4 have shown to produce specific antibodies against IBDV.

Five out of 10 mice injected with pHCV2.5 have shown to produce specific antibodies against HCV. In the same time, 6 out of 9 mice fed with *E. coli* containing pHCV2.5 have shown to produce specific antibodies against HCV.

The above results indicate that the DNA vaccine administered through the two routes (intramuscular injection and feeding) were effective in triggering specific antibodies against the virus. The result was further confirmed by Western blot analysis.

In ELISA analysis, 5 out of 10 mice injected with pHCV2.5 have shown to produce specific antibodies against HCV. In the same time, 6 out of 10 mice injected with pcDNA3.1-ORF5 have shown to produce specific antibodies against PRRSV.

In group 6, the mice were injected with combined DNA vaccine (pHCV2.5 and pcDNA3.1-ORF5), 7 out of 10 mice have shown to produce specific antibodies against HCV. In the same time, 5 out of 10 mice have shown to produce specific antibodies against PRRSV. In addition, 4 mice in this group have shown to produce specific antibodies against HCV and PRRSV simultaneously.

The above results indicate that the combined DNA vaccine administered through intramuscular injection was effective in triggering specific antibodies against both viruses (HCV and PRRSV). The result was further confirmed by Western blot analysis.

The antibody reaction against each virus using a combined immunization was rather equivalent in quality and intensity to that obtained with a single immunization, considering the mouse to

mouse variability. This indicates that the mouse immune system could accommodate multiple antigens and open the way for multipotent DNA vaccine preparations for pigs.

There are at least three conformation dependent and one linear (conformation independent) neutralizing epitopes in IBDV. Two conformation dependent neutralizing epitopes located in the central region of VP2 while the linear neutralizing epitope was located in the C-terminal of VP3 (Yamaguchi, T., *et al.*, 1996, *Virology* 223:219-223). Although Western blot can only detect linear antigen, VP2 was detected on the membrane. It may be due to the incomplete denaturation of VP2 that made it detectable.

In the Western Blot analysis, homodimer and heterodimer of HCV has been detected. Dimerization of HCV protein may be important for authentic antigen presentation to the host's immune system and the induction of a stable, long-term immunity (Konig, M., *et al.*, 1995, *J. Virology* 69:5479-86). In addition, since the viral proteins were separated in non-denaturing condition, E1-E2 heterodimer of HCV that was linked by intermolecular disulfide bridges could be detected. Intramolecular disulfide bonds coated in the N-terminal half of E2 have been shown to be important for antigen recognition by specific antibodies. In order to demonstrate monomeric E1 and E2, the proteins should be separated in reducing condition (*e.g.*, in the presence of β -mercaptoethanol).

Three different forms of glycoproteins E2 were found in HCV, they were E2 monomers, E2 homodimers and E1-E2 heterodimers. E1-E2 heterodimer was preferentially formed after introducing pHCV2.5 that expressed both E1 and E2. The appearance of doublets or triplet of these forms may be due to different E2 protein backbones and to different glycosylation. Moreover, alternative processing at the carboxy terminus generated E2 molecules with different apparent molecular weights.

In Western blot analysis of PRRSV-specific antibodies, there were a number of bands. They may be due to the following reasons. Firstly, the PRRSV protein was heat-treated at 100°C for 10 minutes before loading onto the SDS-PAGE. Hence, three types of GP5 may appear on the gel, i. Totally denatured, ii. Partially denatured, iii. Partially degraded.

Secondly, at around 26.4 kD and 48.6 kD, there was a clear band that may correspond to the disulfide-linked M-GP5 heterodimer. Cysteine residues located at the N-terminal ectodomains of both envelope proteins were probably involved in the formation of an intermolecular disulfide bridge. Hence, the presence of anti-GP5 antibody could detect its presence.

There were at least two types of neutralizing antigenic determinants associated with the GP5 of PRRSV. Among these determinants, some were conformation dependence and some were linear. Therefore, Western blot analysis could still detect the presence of anti-GP5. In addition, it appeared that glycosylation was not necessarily associated with the neutralizing epitopes.

EXAMPLE 4

Contemplated Modifications to Vaccine Compositions and Methods

It is contemplated that certain modifications to the vaccine compositions and methods demonstrated herein may provide additional aspects of the present invention.

Firstly, it is contemplated that a counterpart to each group should be included for comparison. For example, besides feeding mice with *E. coli* containing the plasmid (potential DNA vaccine), an additional group of mice should be fed with naked plasmid DNA. Besides

injecting mice with naked plasmid, an additional group of mice should be included and injected with *E. coli* containing the plasmid. Moreover, instead of injecting or feeding nothing to the mice in the control group, they could be injected with PBS or vector (pcDNA3.1) alongside the vaccination scheme.

Secondly, it is contemplated that the production of IgA and cytokine could be confirmed by analyzing frozen small intestinal tissue sections of the vaccinated mice containing the most Peyer's patches. It has been said that oral immunization can elicit the production of both systemic and mucosal antibodies (Gallichan WS, Rosenthal KL. 1995. *Vaccine*. 13:1589 - 1595).

Thirdly, although the DNA vaccine (pcDNA3.1-VP505.2&VP2-3.4, pHCV2.5 and pcDNA3.1-ORF5) can elicit the production of specific antibodies, as previously demonstrated, it is contemplated that a DNA vaccine may be able to protect the animal against the disease. To further demonstrate the efficacy of the DNA vaccine, the neutralizing ability of the specific antibodies can also be tested by virus neutralization assay.

Fourthly, it has been said that immunization with DNA vaccine can elicit both humoral and cellular immunity (Ulmer, J.B., *et al.*, 1996, *Immunol* 89: 59-67; Ulmer, J.B., *et al.* *Cur Opin Immunol* 8:531-536). However, only the humoral immune response was measured after each vaccination. It is contemplated that T lymphocytes, which play an important role in fighting against the virus, may be stimulated by the vaccine and compositions of the present invention to produce an immune response. It is further contemplated that the quantity and the role of antigen specific cytotoxic and helper T lymphocytes in fighting against a disease, after vaccination using the compositions and methods of the present invention, may be demonstrated by flow cytometric analysis.

Fifthly, it is contemplated that an anesthetized animal may be easier to vaccinate using the compositions and methods of the present invention. For example, during injecting mice with DNA vaccine, a vaccinated animal may contract their muscles and squeeze the vaccine solution (*e.g.*, a DNA vaccine) out when they were awake.

Sixthly, it is contemplated that in order to enhance or boost the immune response of an animal (*e.g.*, mice), one or more additional agents may be used in combination with any of the vaccine methods and compositions described herein. Such agents may include, but are not limited to, one or more chemical (*e.g.*, complete Freund's adjuvant) or genetic (*e.g.*, vector expressing cytokines) adjuvants. In a non-limiting example, co-inoculation of both an immunogenic DNA vaccine (*e.g.*, a plasmid) and a genetic adjuvant (*e.g.*, another plasmid) may result in an augmentation of an antigen specific humoral and/or cell mediated immune response.

EXAMPLE 5

Demonstration of Isolation and Characterization of a Porcine Pathogenic Virus

Described herein this example is methods for identifying, isolating and characterization of a pathogen, specifically guangzhou porcine reproductive and respiratory virus. Specifically described is a method to detect the presence of porcine reproductive and respiratory syndrome virus (PRRSV) in tissue samples of infected pigs, based on reverse transcription of the viral RNA coupled to DNA amplification by polymerase chain reaction (RT-PCR). Tissue samples (lung, muscles) were collected from infected pigs from different pig farms in Guangzhou. AV Guangzhou field isolates described herein have a close similarity with virions of North American strain through the RT-PCR amplification, sequencing analysis, and Western immunoblotting analysis. As would be understood by one of skill in the art, such methods may

be applied in identifying, isolating and characterizing other pathogens in the practice of the present invention.

A. Identification of PRRSV as a New Pathogen

In the late 1980's, a mysterious disease broke out in pig farms throughout the world, characterized by severe reproductive failure in sows of any parity and respiratory problems for pigs of all ages (Mardassi *et al.*, *Can J. Vet Res* 58, 55-64; Mardassi *et al.*, 1994. *J. Clinical Microbiology*. Sept, 2197-2203). The disease was first reported in North American in 1987, and the etiological agent was first isolated in 1990 in Europe and then in 1992 in the USA (Suarez *et al.*, 1994 *Arch Virol*. 135, 89-99). Both strains were structurally related but antigenically distinct. The disease was finally given the name Porcine Reproductive and Respiratory Syndrome disease, caused by a fastidious virus-Porcine Reproductive and Respiratory Syndrome Virus (PRRSV).

B. Characterization of PRRSV

Molecular characterizations have shown that the viral genome consists of a positive single-stranded RNA molecule with an approximate genome size of 15 kb. The genome contains 8 open reading frames (ORFs) with ORF1a and ORF1b (at the 5' end) representing nearly 75% of the viral genome. It encodes for the proteins with the function of polymerase and replicase activities. The other six structural proteins, ORFs2 to 7, are located at the 3' end of the genome (Conzelmann, K.K. *et al.*, 1993, *Virol* 193: 329-339). The spherical, envelope PRRSV has been described with morphological and morphogenetical similarity to members of the arterivirus group, including *equine arteritis virus* (EAV) and *lactate dehydrogenase-elevating virus* of mice (LDV). Virus replication has failed in many different

kinds of primary and established cell lines, but it does replicate in Porcine Alveolar

Macrophages (PAM) and MARC-145 derived from MA-104 monkey cell

(Dea *et al.*, *Ultrastructural characteristics and morphogenesis of Porcine Reproductive and Respiratory Syndrome Virus propagated in the highly permissive Marc-145 cell clone*. Plenum Press, New York. 1995).

C. Isolation and Characterization of the Sub-Strains of PRRSV

In brief, multiplex PCR utilizing different sets of oligonucleotide primers was used to differentiate between North American and European isolates among the field isolated samples collected from Guangzhou. Using purified PRRS virion from the infected cell cultures as the starting material, molecular cDNA cloning and sequencing was also performed. The morphological and physicochemical properties of the field isolate of PRRSV were characterized as well.

1. Cells and virus isolation

A total of 3 Guangzhou field isolates of PRRSV were recovered from clarified lung tissues and muscles of dyspneic pigs and were propagated on MARC-145, a PRRSV permissive cell line (Department of Veterinary Medicine, South China Agricultural University, Guangzhou, 510642, China). The MARC-145 cells were cultivated in Minimum Essential Medium (MEM) supplemented with 10% gamma-irradiated fetal bovine serum (FBS) and 10% Tryptose Phosphate Broth (TPB) and 1% antibiotic-Antimycotic (Life Technologies, GIBCOBRL®). The representative US isolate of PRRSV, NVSL, was isolated from commercially available modified-live vaccine (Animal and Plant Health Inspection Services, National Veterinary Services Laboratories. Ames, IA). Aliquots of 1.5 ml of clarified tissue samples were inoculated into the cell monolayer of the 75 cm² culture flask with culture medium containing

no fetal bovine serum (FBS). Infected cell culture was monitored daily for the appearances of cytopathic effect (CPE).

For all tissue sample homogenates, no CPE was observed during the first six passages in the MARC-145 cell culture. CPE was observed after the seventh passage beginning with the characteristics of rounding off; cell enlargement and rounded cells with many vacuoles appearing after 24 hours postinoculation with the AV field isolates of PRRSV; syncytia of the infected cells was observed; the infected cells started to aggregate after 3-4 days postinoculation. Finally the infected cell sloughed off from the flask. These CPE was also seen on positive control NVSL infected cells throughout the experiment.

2. Virus purification

Stock viruses were produced by at least seven successive passages in MARC-145 cells. The viruses were harvested by freezing and thawing the infected cells three times at -80°C and 37°C respectively. The cellular debris was removed by centrifugation at 4000 x g for 20 min at 4°C. The extracellular virus in the clarified supernatant fluids was first pelleted for 3 hours at 75,000 x g (21,000 rpm) in a Beckman SW40Ti rotor. The pellet was resuspended in 1/100 original volume of TNE buffer. Concentrated virus was then purified through a 30%-50% (w/v) sucrose gradient in a Beckman SW55Ti rotor at 110,000 x g (35,000 rpm) for 16 hours.

3. RNA preparation

Viral RNA was extracted directly from 500 µl of the supernatant from virus infected MARC-145 cells by using 500 µl TRIZOL reagent (Life Technologies INC.,) and following manufacturer's protocol.

4. cDNA synthesis

cDNA synthesis was done according to the Superscript™ Preamplification System for First Strand cDNA Synthesis (Life Technologies, GIBCO BRL).

5. Primer Design

Several sets of primers were designed based on both North American (VR-2332) and European (LV) genome sequences within ORF1b encoding the polymerase protein in order to perform a rapid multiplex PCR assay. PCR primers were designed on the basis of ORF1b due to their conservation among arteriviruses. One set of internal primers was designed from Leiestad virus (LV) genome sequences, while the other set were type-specific internal primers and type-common primers for multiplex or nested multiplex PCR. Primers were designed after sequencing a portion of ORF1b from two North American strains of PRRS virus: Minnesota MN1b and Quebec LHVA-93.3 isolates (Gilbert *et al.*, 1997, *J. Clinical Microbiology* Jan 35(1), 264-267). Details of the oligonucleotide primers for nested multiplex PCR were shown in Table 27.

Table 27		
Oligonucleotide primers for PCR amplification and typing of PRRSV		
Primer Sequence (5' to 3')	Size of PCR product (bp)	Type Detected
Nested multiplex primers		
U1 GTATGAACTTGCAGGATG (SEQ ID NO:17)	186	European
D1 GCCGACAATACCATGTGCTG (SEQ ID NO:18)		
U2 GGCGCAGTGAAGAGA (SEQ ID		

NO:19) D2 GTAACCTGAACACCATATGCTG (SEQ ID NO:20)	107	North American
External primers EU CCTCCTGTATGAACTTGC (SEQ ID NO:21) ED AGGTCCTCGAACTTGAGCTG (SEQ ID NO:22)	255	Common

6. Nested multiplex PCR for PRRSV typing of samples D2, D3 and AV

For the first round of PCR, 2.5 µl of cDNA was added into the mixture containing 10 x PCR buffer, 25 mM MgCl₂; 10 mM dNTPs mix, 12.5 µM primers EU and ED and 5 units of *Taq* DNA polymerase (Life Technologies, GIBCO, BRL) in a total volume of 25 µl. After denaturation at 94°C for 3 min, the reactions were cycled 4 times at 94°C for 20 sec, 42°C for 1 min, and 72°C for 1 min; and then 40 times at 94°C for 20 sec, 47°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 5 min.

For nested PCR, 2.5 µl of the template (PCR product in first round PCR) was added into a reaction mixture containing 12.5 µM primers U1 and D1, and 12.5 µM primers U2 and D2. After denaturation at 94°C for 3 min, the reactions were cycled 35 times at 94°C for 20 sec, 47°C for 30 sec and 72°C for 30 sec with a final extension step of 72°C for 15 min. The amplified products were detected by electrophoresing 5 µl aliquots through 1.5% agarose gel and stained with ethidium bromide, the gels were photographed under UV illumination.

The nested multiplex PCR assay utilizing type-common primers EU and ED in the first round of PCR amplification produced a PCR product being about 255 bp. The U1 & D1 primers

predicted the amplification of a 186 bp product for LV (European genotype) and a 107 bp product for North American strains MN-1b and LHVA-93-3 by using U2 & D2 primers. All the three field isolates resulted in 255 bp products in the first round of PCR amplification, which means that the viruses shared the two genotypes. The 107 bp PCR products were observed in nested multiplex PCR, indicating that the viruses in the tissue samples mostly likely belong to the North American isolate. Markers used for product size measurements included a 1Kb marker and a ϕ X-174 marker.

No amplification was observed for the European strains, thus confirming the specificity of the internal primer pair U2 and D2 for the North American isolates and its use for the differentiation between North American and European isolates of PRRSV

7. Sequencing determination/analysis

The RT-PCR amplified products were purified from a 1.5% agarose gel with Geneclean II Nucleic acid Purification Kit (BiolOI). The genomic region was sequenced on both strands using U2 and D2 primers in an Automated Laser Fluorescent DNA sequencer (PERKIN ELMER, ABI Prism™ 310 Genetic Analyzer). Computer analysis of the nucleotide and sequences was performed using the sequencing analysis 3.4 program as well as MAC DNAsis Version.2.4 software. Nucleotide homologies were also calculated using the MAC DNAsis Version 3.6 program and Geneworks Version 2.2 program (Intelligenetics, Inc., Mountain View, California, USA).

The obtained nucleotide sequences of the ORF1b of the 3 field PRRSV isolates were compared to the published sequences of a reference US strain (ATCC VR-2332) and a reference European strain (LV) as well as the reference NVSL. Table 28 shows no nucleotide substitutions, deletion or insertion among the 3 field PRRSV isolates.

Table 28 Comparison of the nucleotide sequences of the polymerase (ORF1b) gene of the field isolates PRRSV with the sequences of the reference NVSL, European (LV) as well as the US (VR-2332)	
AV-ORF1b	GGCCTGTCGT CCGGCGACCC GATCACCTCT GTGTCTAACA CCATTTACAG TTTGGTGATC TACGCAC-AG CATATGGTGT TCAGTTACAT (SEQ ID NO:23)
D2-ORF1b	GGCCTGTCGT CTGGCGACCC GATCACCTCT GTGTCTAACA CCATTTATAG TTTGGTGATA TATGCAC-AG CATATGGTGT TCAGTTACA-..... (SEQ ID NO:24)
D3-ORF1b	GGCCTGTCGT CCGGCGACCC GATCAGCTCT GTGTCTAACA CCATTTACAG TTTGGTGATC TACGCAC-AG CATATGGTGT TCAGTTACAT (SEQ ID NO:25)
LV-ORF1b	G—TTTTCAT AAAAAAAGGT TAGAAAATAA AAATAAAATC TCCATAGCTG GTGGTTATTT TAAAAGTTAG AATGAGACTA TAAGCAATGG (SEQ ID NO:26)
NVSL-ORF1b	GGCCTGTCGT CCGGCGACCC GATCACCTCT GTGTCTAACA CCATTTACAG TTTGGTGATC TACGCAC-AG CATATGGTGT TCAGTTACAT (SEQ ID NO:27)
VR2332-ORF1b	GGCCTGTCGT CTGGCGACCC GATCACCTCT GTGTCTAACA CCATTTACAG TTTGGTGATC TACGCAC-AG CATATGGTGC TCAGTTACTT (SEQ ID NO:28)

Phylogenetic tree analysis based on the nucleotide sequences on the polymerase gene of the field isolates as well as the reference NVSL, European (LV) and US (VR 2332) isolates was performed. A 17.5% nucleotide homology was observed between the LV-ORF1b strain and the D2-ORF1b, VR2332-ORF1b, NVSL-ORF1b, D3-ORF1b and AV-ORF1b strains; a 95.1% nucleotide homology was observed between the D2-ORF1b strain and the VR2332-ORF1b, NVSL-ORF1b, D3-ORF1b and AV-ORF1b strains; a 95.5% nucleotide homologies of those field isolates (*i.e.*, NVSL-ORF1b, D3-ORF1b and AV-ORF1b strains) was observed with the reference US strain (the VR2332-ORF1b strain, ATCC VR-2332); a 100% nucleotide homology was observed between the NVSL-ORF1b strain and the D3-ORF1b and AV-ORF1b strains; and a 100% nucleotide homology was observed between the D3-ORF1b and AV-ORF1b strains.

The sequencing data of the PRRSV field isolates were used to perform phylogenetic analysis by Unweighted Pair Group Method with Arithmetic Mean (Geneworks Version 2.2).

Accordingly, the field PRRSV isolates were grouped in the North American genotype (the NVSL-1b, Av-1b, D3-1b, VR-2332 1b and D2-ORF1b strains) distinct from the reference European LV strain (LV-ORF1b).

Thus shown herein, RT-PCR can be used for the detection of several RNA viruses. This technique was applied in the present example in order to characterize the strains of the field isolates collected from Guangzhou, so as to confirm the transmission of the new porcine virus from either North America or Europe to Mainland China. It has been indicated that it is a suitable diagnostic procedure not only for detection but also for differentiation between North American and European strains of PRRSV Mardassi *et al.*, *Can J. Vet Res* 58, 55-64; Mardassi *et al.*, 1994. *J. Clinical Microbiology*. Sept, 2197-2203 (1994).

Meulenberg *et al.*, (1993, *Virology* 192, 62-74) showed that most of the PRRSV isolates were able to cultivate in PAM (Porcine Alveolar Macrophages) cells from lung homogenates that supported virus replication. Until recently, propagation of North American PRRSV isolates was achieved in the MARC-145 cells highly permissive cell clones of PRRSV derived from the MA-104 cell line (Kim *et al.*, 1993, *Arch Virol* 133, 477-483). In the present example, virus isolation in MARC-145 was successful, it was found to be permissive to the Guangzhou PRRSV field isolates.

8. Determination of ultrastructural characteristics

Ultracentrifugation of concentrated extracellular virus yielded an opalescent band at the bottom of the tube corresponding to 50% sucrose density gradient. Viruses in the fractions from density gradients were spotted on formav-carbon-coated grids by floating the grid on 30 μ l aliquots of fraction from density gradients for 30 sec and then negatively stained with 3%

aqueous phosphotungstic acid (PTA) pH 6.3 for 30 sec. The grids were examined on a Philips EM 208s electron microscope at different potentials after irradiating the grid with UV light for 15 min. Negative stain electron micrograph of the AV and NVSL reference viral particles observed in sucrose gradient fraction of 1.34 g/ml under different potentials (90 kV and 31.5 kV). Through electron microscopic examination, this gradient fraction contained numerous spherical, enveloped viral particles 60 nm in diameter with cellular debris.

The morphological characteristics of the AV field isolates of PRRSV were also in agreement with previously described tissue culture adapted from ATCC-VR2332 American isolate of the PRRSV (Murphy *et al.*, 1992, *Vet. Microbiol.* 32, 101-115; Pirzadeh, B., Gagnon, C.A. and Dea, S., 1998, *Can J. Vet. Res.* 62, 170-177).

9. Viral polypeptide identification

Sucrose gradient purified preparations (*i.e.*, fractions from 50% to 30%) of the field PRRSV isolates AV and the reference NVSL PRRSV were analysed by SDS-PAGE under non-reducing conditions, and viral polypeptides were deduced from Western immunoblotting experiments using commercially available homologous hyperimmune sera of pigs as the source of specific virus antibodies. Both the SDS-PAGE and Western immunoblotting assay were done according to the Fritsch., Maniatis and Sambrook., 1989. *Molecular Cloning, A Laboratory Manual*. (Second Edition. pp 18.47-18.75). In Western immunoblotting, reference hyperimmune serum (Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, Ames, IA), Alkaline Phosphatase (AP) labeled rabbit anti-porcine IgG (Zymed) and enzyme-substrate solution consisting of NBT and BCIP buffer solution. (Zymed, S. San Francisco, California, USA) were used. The relative migration distances of the bands observed in the gels correspond to approximate MW of 15 kDa, 19 kDa, 26 kDa, 45 kDa and

80 kDa. Only the 15 kDa, 19 kDa, 26 kDa and 45 kDa viral polypeptides were immunoprecipitated using the positive control antisera.

From the result of the Western immunoblotting experiments, it appears that from the bands obtained by SDS-PAGE, only those corresponding to estimated MW of 15 kDa, 19 kDa, 26 kDa and 45 kDa may in fact represent viral proteins. Other bands detected by SDS-PAGE could correspond to cellular proteins co-purified with the extracellular virions. The polypeptide patterns identified for the Guangzhou field isolate AV appear identical to those reported for the American reference isolate ATCC-VR2332 propagated in the continuous CL2621 cell line (Francki *et al.*, 1992, *Arch Virol* . 2, 220-222) as well as the reference NVSL. As mentioned by the others (Plageman *et al.*, 1992, *Adv. Virus Res.* 41, 91-192; Mardassi *et al.*, 1994), these polypeptide patterns are compatible with those determined for EAV and LDV. By analogy to EAV and LDV, the 15 kDa, 19 kDa and 26 kDa polypeptides identified for PRRSV represent the major nucleocapsid protein N, the matrix protein M and the envelope protein E respectively, while the 30 kDa, 31 kDa and 45 kDa polypeptides represent minor structural protein of the viruses.

EXAMPLE 6

Demonstration of Isolation and Characterization of a Chicken Pathogenic Virus

The example herein demonstrates the isolation and characterization of different sub-strains of chicken Infectious Bronchitis Virus (IBV) based on S1 gene diversity. Five regional IBV isolates collected from different geographical region in Southern China were characterized with PCR sequencing. A pair of primer flanking the whole S-1 gene of the IBV was designed according to the published sequence data, and the expected PCR products size is 1760-base pair, The resulting PCR product was further sub-cloned into pGEM-T easy vector and

subjected to sequencing. The analysis of the genetic relationship among the isolates indicates the diversity of S-1 gene of the 5 isolates in Southern China was very high. The nucleotide variation among these 5 isolates was ranging from 8% to 48%, and the 5 isolates could be classified into 3 groups according to the phylogenetic tree analysis. Two conserved regions and two hyper-variable regions were also identified among these isolates. This example indicates chicken farms at different regions of China should vaccinate their chicken with their respective genotype matched vaccine strains of the particular region so as to prevent failure of vaccination. Additionally, other pathogen substrains can be identified for more effective vaccine preparation and administration in accordance with the present invention.

A. Viruses Collection

Field IBV isolates V1, V2, V3, V4 and V5 were obtained from Yunnan, Hunan, Hubei, Guangxi, and Guangdong provincial veterinary service station of China.

B. Virus Isolation

Each isolate was propagated and titrated in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs at 37°C for 48 hr 500 microliters of allantoic fluid was collected from inoculated embryos and centrifugated at 2500 x g for 5 min at 4°C. After centrifugation, the supernatant was collected, IBV genomic RNA was isolated according to manufacturer's instructions (Gibco BRL, Grand Island, NY).

C. RT-PCR

Viral RNA was used as a template to reverse transcribe the first strand cDNA. The superscriptase RT kit was used per manufacture's instructions (Gibco BRL, Grand Island, NY). cDNA was synthesized from 1 µl (200 ng) viral RNA primed with random hexamers. Amplification of cDNA was performed in a volume of 25 µl that included 2.5 µl 10 x PCR reaction buffer, 0.5 µl 10 mm dNTP mix, 0.5 25 mm MgCl₂, 1 µl cDNA, 1 µl Taq DNA polymerase and 50 pmole of each primer. Adjust volume to 25 µl with distilled water. PCR amplification was performed for 35 cycles (94°C 1 min, 52°C 2 min, 72°C 2 min), with a final elongation step of 10 min at 72°C. Using a robocycler PCR apparatus.

S1 gene was amplified by using a 1760-base pair primer. All the isolates yielded a 1760-base pair fragment with 1760-base pair primer. A Lambda DNA/*Hind*III marker was used to size the clone fragments. This result confirmed all isolates were IBV. The isolated were designated V1, V2, V3, V4 and V5 respectively.

D. Cloning and Sequencing S-1 Gene of the 5 Isolates

The S-1 gene PCR products were purified by using a Geneclean II kit (101 Bio. Co), and cloned into the plasmid pGEM-T easy vector (Promega) according to the manual. Plasmid PCR and *Eco*RI digestion was used to confirm the right clone (pT-S). A Lambda DNA/*Hind*III marker was used to size the clone fragments. The clone was sequenced by primer walking strategy, primer and its position was listed in Table 30 and the resulting sequence of the isolates were compared by MacDNAsis and PAUP (Hitachi software engineer Co. Sun brew, CA).

Table 30

Primers used for PCR and sequencing

Primer	Sequence	Strain	PCR Product	Position
Primer-1	5' CCGAATTCGCTATGAAAAGTGAACAAAA 3' (SEQ ID NO:29)	+	1760 bp	-124
Primer-2	5' GGGTCGACATCCATAACTAACACAAGGG 3' (SEQ ID NO:30)	-		1636
IBV-F	5' TCAAAGCTTCANGGNGCNTA 3' (SEQ ID NO:31)	+	573-601 bp	127
IBV-R	5' CTCGAATTCNGTRTAYTGRCA 3' (SEQ ID NO:32)	-		708
IBV-FOR	5' GTATTCTGCTTTAAAAAG 3' (SEQ ID NO:33)	+	788-800 bp	395
IBV-REV	5' AGCTCACC ACTATAAACA 3' (SEQ ID NO:34)	-		1183

The whole sequence of S1 gene was obtained by sequencing with three primer pairs (primer-1 and primer-2; IBV-F and IBV-R; IBV-FOR and IBV-REV). In order to get the whole sequence of S-1 gene, a 800-base pair fragment of S-1 gene for the V1, V2, V3, V4 and V5 isolates was RT-PCR amplified, and three positive clones subcloned into pGEM-T easy vector.

Nucleotide sequences in the N-terminal region of the S-1 gene (Table 31) revealed two hypervariable regions and two conserved regions identified by Keeler *et al.*, in the five isolates.

Table 31

S-1 gene sequence differences

	CK4 Positions 43 to 47	HV1 Positions 54 to 68	HV2 Positions 116 to 141	CK2 Positions 229 to 236
D41	HGGAY (SEQ ID NO:35)	.SENNAGTAPSCTAG (SEQ ID NO:36)	.KSGSNSCPLTGLIPKGQIRISAMR- SVNSRLHI (SEQ ID NO:37)	..CQYNTG (SEQ ID NO:38)
V5	HGGAY	.TISYNAGTA..CTAG (SEQ ID NO:39)	.KAGSN..GLIPKGQIRISAMR-SVNSRLHL (SEQ ID NO:40)	..CQYNTG
Beaudette	HGGAY	.SENNAGTAPSCTAG (SEQ ID NO:41)	.KSGSNSCPLTGLIPKGQIRISAMR- SVNSRLHF (SEQ ID NO:42)	..CQYNTG
V3	HGGAY	.SENNAG-APSCTAG (SEQ ID NO:43)	.KSGSNSCPLTGLIPKGQIRIRMASVNSRLTI (SEQ ID NO:44)	..CQYNTG
Holte	HGGAY	.SENNAGTAPSCTAG (SEQ ID NO:45)	.KAGSNSCPLTGLIPKGQIRISAMR- SVNSRLHI (SEQ ID NO:46)	..CQYNTG
V2	HGGAY	.SENNAPTQY.SCTAG (SEQ ID NO:47)	.KSGSN..CPLTGLIPKGQIRISAMR- SVNSRLF (SEQ ID NO:48)	..CQYNTG
V4	HGGAY	.TISY..TAPSCT----AG (SEQ ID NO:49)	..ISGSNSCPLTGLIP..RISAMR-NVNSRLLI (SEQ ID NO:50)	..CQYNTG
V1	HGGAY	.SEINAG.....SCTAG (SEQ ID NO:51)	.KAGSNSC..LIPKGQIRISAMR-SVNSRLHA (SEQ ID NO:52)	..CQYNTG

E. Genotype Analysis

Phylogenetic tree analysis based on the comparison of S-1 nucleotide sequences of 8 IBV strains was conducted. The five IBV field isolates collected from Southern China were highly heterogeneous on the basis of nucleotide sequences (range from 48%-8%). V4 and V5 were categorized as belonging to the same group, and is closer genetically due to a high homology (92%) with the Holte strain than the mass-2 (Beaudette strain). V1, V2 and V3 were classified into different groups. V1 belongs to a new type. V2 and V3 also showed some homologous with Holte, but there was also a big variation between them. The D41 was highly homologous with mass-2 (Beaudette strain).

Two hypervariable regions 56-69 and 117-133 and two conserved regions 43-47 and 229-236 in the N-terminal amino acid residues of the S-1 gene of Mass-typed IBV had been recognized previously. Both hypervariable regions and conserved regions were identified among the 5 isolates. The results indicate these isolates were mass-typed. However, the phylogenetic tree analysis showed the isolates had some difference with mass-typed strain. Therefore, it is contemplated that the mutation of mass-typed vaccine had lead to the emerging of field virulent IBV in Southern China.

The demonstrated high diversity among these isolates indicate that chicken farms at different regions of China may vaccinate their chicken with their respective genotype matched vaccine strains of the particular region. It is contemplated that such genotyping of pathogens (*e.g.*, IBV) may allow genotype specific, the production and utilization of the vaccines and methods of the present invention.

EXAMPLE 7**Demonstration of an Immunization of a DNA Vaccine Against Hog Cholera Virus**

The example herein demonstrates the immunization of DNA vaccine with improved efficacy. To demonstrate an improved efficacy of a DNA vaccine against hog cholera virus, a formulated crude DNA vaccine was prepared. After preparation and characterization, the crude bacteria DNA vaccines were administrated to experimental animals and immune responses induced were compared with immunization with naked DNA. A significant improvement in immunogenicity over naked DNA was achieved for both antibody and CTL induction. Specifically, a bacteria DNA vaccine against hog cholera virus induced significantly enhanced serum antibody responses (humoral) and cytotoxic T lymphocyte (cell-mediated) responses in comparison to naked DNA after i.m. immunization in rabbit.

A. Plasmid DNA.

Immungenic DNAs pHCV2.5 and pHCV1.25, constructed with the cytomegalovirus promoters that drive expression of the glycoprotein E2 gene of HCV, have been described herein.

B. Immunization and Vaccine Formulation.

To induce immune responses against HCV, animals were immunized with the following protocol. Female rabbit (1 kg –2 kg) were purchased from animal center of Hong Kong University. Rabbit were fundamentally immunized with a single intramuscular injection into the right biceps femurs muscle of 0.1 ml and 0.5 ml crude bacteria preparations, 0.1 ml formulated vaccine is equal to 100 µg naked plasmid DNA and so on. Booster occurred twice

at 7 days interval. The formulated vaccine is prepared as follows: Inoculate 1 ml bacteria seeds that are propagated from a purified colony to 1.5l LB broth added with 1 mg/ml ampicillin. Incubate at 37°C and shaken with 200 rpm overnight. Spin down and get the bacteria pellet. Weighing and reconstitute to a given concentration, sonication for 10 min, and then add 1 mg/1 ampicillin overnight for administration.

C. Anti-DNA Antibody Responses (ELISA)

An enzyme-linked immunosorbent assay (ELISA) kit was used to determine antibody responses against HCV according to the manufacturer's manual (IDEXX-Co). The rabbit inoculated with a formulated DNA vaccine had a highest ELISA titer among all the rabbits two weeks later; it is even higher than commercial vaccine. Compared with the NA parameters, the data matched.

D. Flow Cytometry

Preparation of single cell suspensions (Peripheral blood PBLs). Whole-blood samples were collected by cardiac puncture into heparinized syringes and placed on ice. The blood was diluted in Han's balanced salt solution (HBSS) without phenol red plus 1.5% fetal bovine serum (Sigma Chemical Co. St. Louis, Missouri) and sieved through a 15 ml rayon wool column primed with HBSS. Five ml of the filtered blood was layered over 5 ml Ficoll Hypaque (Sigma) and centrifuged at 2000 rpm for 10 min. The interface buffy coat was removed with a Pasteur pipette and rinsed three times with 5 ml HBSS. After the final rinse, total lymphocyte from 10^4 cells per sample was determined by analysis on a flow cytometric apparatus.

Virus neutralization test for HCV CSFV neutralizing antibodies (VNAb) were titrated by the rapid fluorescent focus inhibition test (RFFIT) with modification, PK15 was used for the detecting of VNAb against CSFV. Anti CSFV VNAb titers are expressed in serum dilution. Using CSFV C strain as the reference or as the reciprocal serum dilution (rd) that inhibited 50% of the fluorescent focus.

E. Fever Response in Rabbit after a Challenge Test

Based on the principle of virulent HCV can not cause fever of rabbit under normal circumstances, while laprinized HCV vaccine can cause fever in the rabbit. Therefore, it was contemplated that should formulated DNA vaccine could induce immune response in rabbit, it will neutralize the vaccine strain inoculated thereafter, and there will be no fever response.

Five rabbits (2 kg-2.5 kg) were used in this demonstrated. Rabbit No. 1 and No. 2 were injected with high dose and low dose formulated DNA vaccine, No. 3 and No. 4 were injected with naked plasmid DNA and conventional vaccine respectively, No. 5 were set as control.

Firstly, each rabbit was primed with above materials on day 0. After rabbits were intravenous inoculated with a laprinized vaccine, formulated vaccine and naked plasmid DNA respectively, and the last two was boosted after 2 weeks. Then, each rabbit was boosted twice at 1 week intervals. Then the rabbits were challenged with the laprinized vaccine strain two weeks latter, and the body temperature determined for each rabbit every day for 1 week.

From the temperature curve, control rabbit had a progressive fever, rabbit No. 3 immunized with the naked plasmid DNA had a fever 4 days later, while rabbit inoculated with formulated vaccine both low dose and high dose had no fever response. The results indicated formulated DNA vaccine had the same efficiency as the HCV laprinized vaccine, while naked plasmid DNA could delay fever 4 days, compared with a formulated vaccine, it only had partially protective capability.

F. Histopathological Examination of the DNA Vaccine-Injected Muscle

7 days after challenge, muscles of rabbit were resected and fixed with 10% buffered formalin and were embedded in paraffin. Sections were directly used for light microscopic observation. Rabbit injected with naked DNA and conventional commercial vaccine was set as control.

A histopathological section injected with formulated vaccine showed massive accumulation of mononuclear cells, indicating that strong inflammation was caused by the injection, furthermore, destructive changes of the muscle fibers associated with severe inflammatory cell recruitment were noted. While in the muscle injected with naked DNA and conventional commercial vaccine, the degree of mononuclear cell infiltration and muscle fiber change were distinguishably mild.

G. Formulated DNA Vaccine in Humoral Immunity (VN)

When rabbit was inoculated with a bacteria preparation of HCV DNA vaccine (pHCV2.5), Both of the rabbit inoculated with low dose or high dose had a humoral response two weeks later, after booster. The VN titer continued to increase, it still remained at a high level 1 week

after challenge. Compared with the naked plasmid DNA and conventional commercial vaccine, the response of neutralization antibody in the rabbit inoculated with the formulated DNA vaccine was even better over them.

H. Formulated DNA Vaccine in Cell-Mediated Immune Response

The un-clotted blood of the rabbit was analyzed by using a flow cytometry, lymphocyte, monocyte and granular cell of the peripheral blood was counted respectively. The results showed rabbit immunized with the formulated DNA vaccine had a highest concentration of inflammatory cells, especially the granular cell. Rabbit No. 2 immunized with a high dose of formulated DNA vaccine had a higher concentration of granular cells, it reflects the non-specific immune response of the rabbit had been greatly enhanced. The rabbit injected with a low dose of formulated DNA vaccine had a relatively low concentration of these cells.

I. Bacterial DNA Vaccine Properties


This example demonstrated that a bacterial formulated DNA vaccine had a better effect over naked DNA vaccine. Superior parameters of Ab titer (ELISA), neutralization activities, cellular immune response and fever response were demonstrated for the bacterial DNA vaccine.

Histopathological examination of inflammatory cells and formulated DNA vaccine injected muscle revealed massive accumulation of inflammatory cells and segmental degeneration and necrosis of myocytes. These characteristics of the DNA bacteria vaccine indicate development strategies for effective DNA vaccine construction, preparation and administration. For example, in immunized rabbits with formulated DNA vaccine will

cause a cellular immune response. The higher the inoculation dose, the higher immune response it will cause; demonstrating superior properties than the commercial vaccine. It is contemplated that these characteristics may be this is due to the bacteria protein or bacteria sequence. In contrast, the effect caused by naked DNA was not so significant as formulated DNA vaccine. Moreover, it is contemplated that the rabbits had some endurance to the endo-toxin of bacteria, and/or the long term treatment to the bacteria vaccine preparation by ultrasonic techniques produced reduced toxicity, since the bacteria vaccine preparations didn't cause fever or death and clinical symptom to the rabbit. Additionally, there was no gross lesion in the injection site, and the rabbit injected with a low dose had been well absorbed, only the rabbit injected with a high dose had some tyromatosis, indicating it had a strong inflammation response once before.

EXAMPLE 8

Demonstration of a DNA Vaccine against a Regional strain of PRRSV

 This example demonstrates a DNA vaccine in the generation of both humoral and cellular immune responses and the ability of a crude bacteria formulation to act as an adjuvant. Specifically, this example demonstrates the immunogenicity of the DNA plasmid constructs in pigs and the use of the ORF 5 polypeptide of PRRSV for specific recognition by T cells to elicit cell-mediated immunity.

An understanding of the humoral immune responses to structural proteins of PRRSV following vaccination is useful for development of an effective vaccine strategy. Furthermore, the production of recombinant vaccines depends on selection of a viral protein that will induce the proper antibody response to protect the animal from disease. The development of humoral immune responses described herein was monitored by ELISA, viral neutralization (VN) and

immunoblotting assays (Nelson E.A., *et al.*, (1994). *J. Veterinary Diagnostic Investigation* 6, 410-415). The role of cell-mediated immunity (CMI) in protection against viral diseases had been widely documented. Most notably, it has been shown that cytotoxic T cells are critical for viral clearance of a number of viruses. The identification of epitopes recognized by T cells have proven to be useful in the design of subunit vaccines for induction of effective immune responses to various microbial pathogens. Previous studies have demonstrated that infected pigs develop CMI responses to PRRSV (Bautista E.M., *et al.*, (1996). *Archives of Virology* 14, 1357-1365) However, it was unknown whether PRRSV polypeptides differ in their ability to elicit T cell immune responses in swine.

PRRSV ORF 5 envelope protein and ORF 6 matrix protein regions were chosen for the production of recombinant construct as a DNA vaccine. As mentioned (Pirzadeh B. and Dea S. (1997). *J. General Virology* 78, 1867-1873), PRRSV GP has a role in virus infectivity and may function in attachment to cell receptors and/or in virus penetration into the cytoplasm of target cells. It is indicated that at least one neutralizing antigenic determinant is associated with PRRSV GP. Since PRRSV GP, is rather abundantly present in the virion and is partially exposed in association with the lipidic envelope (Mardassi H., *et al.*, (1996). *Virology* 221, 98-112; Meulenberg J.J.M, *et al.*, (1995). *Virology* 206,155-163), it shows that GP, is the major viral envelope glycoprotein being recognized by most convalescent pig sera (Meulenberg J.J.M, *et al.*, (1995). *Virology* 206,155-163; Nelson E.A., *et al.*, (1993). *J. Clinical Microbiology* 31, 3184-3189). Then in the case of ORF 6 matrix protein, Bautista (Bautista E.M., *et al.*, (1999). *Archives of Virology* 144, 117-134) indicates that the greater T cell proliferation response induced by *in vitro* stimulation with the product of ORF 6, as compared to the other polypeptides of PRRSV. It indicates that the matrix protein may have a major role in cell mediated immunity to PRRSV. The matrix polypeptide gene is the most conserved gene among all the PRRSV isolates tested (Kapur V., *et al.*, (1996). *J. General*

Virology 77,1271-1276; Meng X.J., *et al.*, (1995). *Archives of Virology* 140, 745-755; Meng X.J., *et al.*, (1995). *J. General Virology* 76, 3181-3188; Meng X.J., *et al.*, (1996). *J. General Virology* 77, 1265-1270; Meng X.J., *et al.*, (1994). *J. General Virology* 75, 795-801; Murtaugh M.P., *et al.*, (1995). *Archives of Virology* 140, 1451-1460), indicating that the structure of this polypeptide may be essential for the assembly of PRRSV. The matrix protein of PRRSV was then selected for a vaccine of the present invention to demonstrated immunization.

The genomic regions encoding ORFs 5 and 6 of the regional strain PRRSV were selected and cloned into the mammalian expression vector pcDNA3.1(+) to construct a DNA vaccine. DNA immunization with plasmid encoding GP5 or ORF 6 of PRRSV, under the control of a human cytomegalovirus promoter, induced anti-GP5 neutralizing antibodies or ORF 6 specific antibodies in both Balb/c mice and its natural host, pigs. The GP5 protein specificity of neutralizing sera was confirmed by immunoblotting, ELISA as well as neutralization assay. This result indicates that neutralizing epitopes for this regional strain PRRSV is present on the viral envelope glycoprotein only.

Inoculation of crude bacteria conjugated with ORF 5-plasmid construct resulted in a stronger antibody level than with plasmid construct alone as shown in the ELISA test. In addition, cellular immune response was detected in immunized mice and pigs by flow cytometrically counting the increase in number of T cells (CD4⁺ and CD8⁺ populations. It was demonstrated that ORF 5 plasmid construct with crude bacteria as adjuvant may be a used as a vaccine against this regional PRRSV.

A. Animals

Seven-week-old male Balb/c mice were purchased from the Laboratory Animal Unit of the University of Hong Kong and separated in groups of five mice per cage. Mice were randomly divided into seven experimental groups. Eight piglets weaned at 5 weeks of age were obtained from a breeding farm in the Department of Veterinary Medicine, South China Agricultural University, Guangzhou, 510642. The breeding piglets were tested and proven to be seronegative for PRRSV. The piglets used in this example were randomly divided into four groups.

B. Virus

The regional strain AV Guangzhou field isolate PRRSV was initially isolated from tissue samples of PRRSV infected pigs in Guangzhou and propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV (Kim H.S., *et al.*, (1993). *Archives of Virology* 133,477-483). Virus titers were expressed as tissue culture infective dose 50 (TCID₅₀) per ml, as previously described (Dea S., *et al.*, (1992). *Canadian Veterinary Journal* 33, 801-808).

C. Recombinant Constructs

Viral RNA was extracted from AV strain PRRSV-infected MARC-145 cells as previously described (Mardassi H., *et al.*, (1995). *Archives of Virology* 140,1405-1418). Oligonucleotide primers enabling the amplification of PRRSV ORFs 5 and 6 were designed based on the-nucleotide sequence of the genome of the American strain VR-2332. *Hind*III or *Xba*I restrictions (RE) sites for ORF 5 region and *Bam*HI or *Eco*RI for ORF 6 region were incorporated at the 5' end of the primers to facilitate cloning. The ORF 5 and ORF 6 regions

were first cloned into the TA-cloning vector (pGEM®-T and pGEM®-T Easy Vector Systems Promega) and then directly inserted into specific RE sites of the pcDNA3.1(+) expression vector (Invitrogen) and resulted in the production of pcDNA3.1-ORFs 5 and 6 constructs. Recombinant constructs were verified by sequencing and then transformed into *E. Coli* strain, Top-10. Bacterial culture pellet was dried and sonicated in preparation for injecting the animals.

D. Transient Expression of the Recombinant Proteins

In vitro expression of the pcDNA3.1(+)-ORF 5 or 6 constructs were tested in transient expression experiments in HEK-293 cells maintained as confluent monolayers. Cells in 6-well tissue culture plates were transfected with 5 µg plasmid DNA by liposome. For immunofluorescent staining, cells were incubated at 37°C and fixed with 37% formalin for 15 minutes at room temperature at various times (18 hours-72 hours) post-transfection. The monolayers were then reacted for 1 hour with anti-PRRSV polyclonal antibody (National Veterinary Services Laboratories, Ames, IA) and the immune reaction was determined following incubation with FITC-conjugated secondary antibody (Zymed).

Transient expression of the cloned ORF 5 and ORF 6 gene were detected by immunofluorescent staining. Expression of the protein product was demonstrated in HEK-293 cells at 72 hours post-transfection. The identification of the protein product was confirmed by using porcine anti-PRRSV serum. Secondary antibody with FITC-conjugated was added to give signals to the transfected cells. Green color fluorescent signals were detected under fluorescence microscopy. The transfected cells showed bright homogenous cytoplasmic reaction by using polyclonal antibody and the expressed proteins tended to accumulate near the perinuclear region. In order to confirm the expressed proteins represented the PRRSV envelope

protein and the matrix protein, Western immunoblotting was carried out using the porcine anti-PRRSV serum. Specific protein bands with size 26 kDa and 19 kDa were observed in either lane, which corresponded to envelope and matrix protein respectively.

E. Mice Immunization Schedule

In vivo expression of pcDNA3.1-ORF 5 or 6 was verified by immunizing groups of five Balb/c mice with 500 µg of either ORF 5 or ORF 6 construct or 100 µg of ORF 5 construct; a mixture of 100 µg of each construct or crude bacteria with 50 µg or 10 µg of ORF 5 construct plasmid equivalent diluted in 250 µl PBS. DNA was injected into the tibialis cranialis muscle with a 27-gauge needle. The mice were boosted twice with the same quantities of DNA at 2-week intervals. Control mice received 100 µg of pcDNA3.1 (+) vector via an identical route.

Following DNA inoculation, the protein specificity of mouse sera to GP5 or ORF 6 was established by ELISA and by immunoblotting with expressed proteins. Antibodies against PRRSV antigen in the ELISA were tested positive at various time post-immunizations, indicating a humoral immune response had been generated. In general, antibodies specific to PRRSV were increased in all mice after the first booster of the constructs (day 0). The second booster occurred on day 14. Antibody titer rose to maximum value by 21 days post-immunization (PI) for groups injecting with DNA plasmid constructs and then began to decline. But for groups injecting with crude bacteria with plasmid equivalent, antibody titer rose to maximum value by days 14 PI and remained at a steady high level. Serum neutralization assay was performed using day 35 mice sera. Sera from three of the four mice inoculated with pcDNA3.1-ORF 5 and one of the three mice inoculated with mixture of pcDNA3.1-ORF5 and pcDNA3.1-ORF6, together with four of the five mice inoculated with crude bacteria pcDNA3.1-ORF 5 plasmid equivalent demonstrated *in vitro* neutralizing activity

at day 35 of PI. Neutralization was not observed in mice injected with pcDNA3.1-ORF 6, or sera obtained from the vector-injected control mice and pre-immune sera. These results indicate that PRRSV-neutralizing antibodies were specifically targeted to the epitopes of proteins encoded by ORF 5 of PRRSV.

F. Pig Immunization Schedule

Two piglets in each group were injected three times at 2-week intervals with 100 µg ORF 5 plasmid construct or crude bacteria with 100 µg or 500 µg of ORF 5 plasmid equivalent diluted in 5 ml PBS. PIG 1 and PIG 2 were administered 100 µg ORF 5 plasmid construct; PIG 3 and PIG 4 were administered 100 µg control vector pcDNA3.1; PIG 5 and PIG 6 were administered crude bacteria with 100 µg ORF 5 plasmid equivalent; and PIG 7 and PIG 8 were administered crude bacteria with 500 µg ORF 5 plasmid equivalent. Control piglets received 100 µg of pcDNA3.1 (+) vector via an identical route. Two-thirds of the volume was injected, using 22-gauge needle, into the tibialis cranialis muscle of the leg and one-third was intradermally administered into the dorsal surface of the ear. The first booster was administered day 0, the second booster was administered day 14 and the third booster administered day 28. Preimmune and hyperimmune sera and blood lymphocytes were collected from all mice and pigs prior to each plasmid inoculation to evaluate their immune responses.

Seroconversion was also demonstrated in pigs by ELISA whereas the protein specificity of pig sera to GP5 was established by immunoblotting with samples taken 35 days post-inneculation. All pigs developed antibody after immunization, but pigs injected with crude bacteria with ORF 5 plasmid equivalent (PIG 5-8) showed a stronger antibody response than pigs injected with ORF 5 plasmid construct only (PIG 1-2). Each pig may show variation in response

towards vaccination, only PIG 6 and 7 developed a faster antibody response after the third booster, but only PIG 7 was able to maintain a high-level antibody level for about two weeks, then started to decline. Neutralizing antibodies were detected in sera of the DNA-immunized pigs only 2 weeks after the second booster injection and *in vitro* neutralizing activities still maintained until the end of the experiment. Thus, the ability of the DNA vaccine to produce a humoral immune response in another animal model (*i.e.*, pigs) was demonstrated.

G. Virus Neutralization and Serological Tests

Under some circumstances, as in vaccination, it may be desirable to enhance the normal immune response by administering an adjuvant with the antigen. Adjuvants enhance the body's immune responses to the antigen. A large variety of compounds have been employed as adjuvants. In the type of bacterial fractions, endotoxins enhance antibody formation if given about the same time as the antigen. They have no effect on delayed hypersensitivity, but they can break tolerance, and they have a general stimulatory activity. Endotoxins act by stimulating macrophage production of interleukin-1. Thus the use of crude bacteria as adjuvant shows substantial facilitating effects on the antigen-specific serum and antibody responses. To determine if the anti-ORF 5 and anti-ORF 6 protein monoclonal antibodies could neutralize virus infectivity, virus neutralization assay test is done on MARC-145 cells in the presence of $10^{6.5}$ TCID₅₀ of the virus.

Following DNA inoculation, humoral immune responses in each animal are monitored by ELISA and immunoblotting prior to the detection of antibody by the virus neutralization assay test. The virus neutralization assay test is less sensitive than the other tests; it could be affected by circulating immune complexes, or neutralizing antibody may actually not appear until later after initial infection. Also, the antigenic epitope(s) associated with virus neutralization may

represent only one of many antigenic epitopes on a viral protein. Therefore, the immunoblotting assay detects antibody prior to the appearance of neutralizing antibody.

Mouse and pig sera were tested for the presence of specific anti-GP5 or ORF 6 antibodies by virus neutralization (VN), ELISA and Western immunoblotting tests. The VN tests were performed in duplicate with 100 μ l serial dilutions of virus ($10^{6.4}$ TCID₅₀) in the presence of 50 fold dilution of heat-inactivated test sera, incubated for 60 minutes at 37°C. The mixtures were put in contact with confluent monolayers of MARC-145 cells seeded in 96-well plates 48 hours earlier. Cell monolayers were incubated at 37°C and observed daily for the appearance of cytopathic effect (CPE). Neutralizing index was expressed by subtracting the reciprocal of log₁₀ highest virus dilution liter showing no CPE with test serum from log₁₀ liter with non-immune control serum. Humoral immune responses in each animal were monitored by ELISA using the PRRSV antibody test (IDEXX HERDCHEK-PRRS, Westbrook, ME) and Western blot assays were also performed (Pirzadeh B. and Dea S. (1997). *J. General Virology* 78, 1867-1873) using ORF 5 expressed protein as antigen.

In vivo expression of foreign proteins via simple injection of plasmid DNA with or without crude bacteria conjugate into mice and its natural host was demonstrated. It was found that circulating antibodies to PRRSV are detected on 14 days PI after the first dose booster by the ELISA test, whereas antibodies could still be detected by ELISA for the entire sampling period. Interestingly, it was found that those groups of animal injected with crude bacteria as adjuvant showed a higher antibody level and maintained at a steady high level after the second booster rather than declining as in other groups with only plasmid injection. The lower titers of antibodies in DNA-immunized mice and pigs as compared to the crude bacteria-ORF 5 immunized animals could be explained by the fact that the injected antigens are available to the

B cells and other antigen-presenting cells which can potentially stimulate a strong antibody response.

These results demonstrate the presence of neutralization epitopes on proteins encoded by ORF 5 but not on ORF 6 encoded proteins. ORF 5 of PRRSV is has been described to elicit neutralizing antibody in pigs and Balb/c mice (Pirzadeh B. and Dea S. (1998). *J. General Virology* 79, 989-999). It is important to note that neutralizing antibodies can only be detected with the association of the conformational neutralizing epitopes on PRRSV protein. DNA constructs, pcDNA3.1-ORF5 and ORF6, has an advantage in that expression in mammalian cells which mimic proper viral protein conformations. These constructs thus have the ability to drive antigen production in host animals resulting in immune responses that recognize native PRRSV antigens in a relevant and protective fashion (Kwang J., *et al.*, (1999). *Research in Veterinary Science* 67, 199-201).

H. Flow Cytometric Analysis of Peripheral Blood Lymphocytes (PBL)

Peripheral blood lymphocytes were prepared from heparinized blood. 0.2 ml of fresh whole blood was added into 5 ml of freshly prepared red blood cell lysing buffer (0.8% ammonium chloride, 0.083% sodium hydrogencarbonate and 0.003% EDTA-free acid, pH 7.3) using PYREX Borosilicate glass disposable culture tube (CORNING, NEW YORK) until complete lysis of red blood cell. Lymphocytes were collected by centrifugation at 2000 rpm for 2 minutes. Lymphocytes were washed with 1 ml of 1 x phosphate buffered saline (PBS) by vortexing the tube and spun down at 2000 rpm for 2 minutes. Lymphocytes were then stained with appropriate FITC conjugated CD4 or R-PE conjugated CD8a (BD PharMingen) at the recommended concentration (1 μ g per 1×10^6 cells) in darkness for 1 hour with constantly shaking. Before running the analysis in flow cytometer (EPICS ELITE ESP COULTER), 0.5

ml of wash buffer (0.1% sodium azide, 1% Bovine Serum Albumin BSA in 1 x phosphate buffered saline PBS) was added into each tube to top up the volume. At least 5000 cells were analyzed for each sample.

The ability of the DNA vaccine constructs to induce a cell-mediated immune (CMI) response was determined by estimating the increase in CD4⁺ and CD8⁺ T cells population at various times of post-immunizations in mice. Boosters for tests involving measurement of CD4⁺ were given on days 0 and 14. A negligible amount of lymphoproliferation in response to mice immunized with control DNA plasmid. 83% of the immunized mice demonstrated cell mediated immune responses. Most prominent was the change in the percentages of CD4⁺ T cells, which peaked on day 14 and 25 of PI at roughly 50% and 42% respectively above the level of day 0. Whereas in the percentages of CD8⁺ T cells, which peaked on day 14 and 28 of PI at roughly 52% and 37.5% respectively above the level of day 0.

On day 14 of PI, mice of group 5 inoculated with mixture of both constructs induced the strongest increase in percentages of both T cells. High percentages of T cells also showed in mice of group 4 inoculated with ORF 6 construct. These results indicated the ability of ORF 6 protein to induce higher T cell proliferation response.

In pigs, boosters for tests involving measurement of CD4⁺ were given on days 0, 14 and 28. Peripheral T cell populations of growing pigs showed then-numbers to drop transiently at day 14 post-immunization (PI) except CD8⁺ T cell populations in FIG 1. Further analysis on the overall trend of the T cell populations, it found an increase in CD4⁺ T cell populations, with a decrease in CD8⁺ T cell populations or vice versa. This observation was shown most significantly in FIG 1 and FIG 2. The decline in CD8⁺ T cell populations (a marker for

cytotoxic T cells, which recognize vims-infected cells) continued for at least 3 weeks, whilst CD4⁺ cells (which include T-helper cells, involved in immunological memory) increased, peaking range from days 28-42 in different pigs. This result may confirm the ability of the PRRSV to suppress the immune system of its host as this result was not shown in mice.

Another characteristics of porcine T cells included a reversal of the CD4/CD8 ratio of T cell subsets, *i.e.*, the population of CD8⁺ T cells is higher than CD4⁺ T cells in porcine, which was vice versa in human and mice. Moreover, the porcine T cell population was unique in that there was a large percentage of CD4⁺CD8⁺ dual expressing peripheral T cells, the percent of this dual expressing cells also increased with the age of the pigs. The result was evaluated in two-color flow cytometric analysis.

DNA constructs induced *in vivo* CMI responses by estimating the number of the individual T cells population *i.e.*, CD4⁺ and CD8⁺ T cells. It demonstrated that during DNA constructs immunization in mice a strong influx of both CD4⁺ T cells and CD8⁺ cytotoxic T lymphocytes in the peripheral blood. This finding strongly indicates that the presence of cytolytic cells in the peripheral blood during primary immunization is protective. Cytotoxic T cells are potent at lysis of infected cells and may thus prevent spread of the virus (Samsom J-N., *et al.*, (2000). *J. General Virology* 497-505).

Furthermore, this type of cells has been shown to regulate cellular immunity via the production of interferon- γ whereas CD4⁺ T cells implicated in eliciting an antibody response.

On the contrary, peripheral T cell populations of growing pigs show their numbers to drop transiently at 14 days post-immunization. During the sampling period, it shows an increase in

CD4⁺ cells and a decrease in CD8⁺ cells in young pigs or vice versa. An increase level of proliferation response after secondary exposure was due to CD4⁺ cells that were effectors in this response. Many theories have been offered proposing a mechanism whereby PRRSV may alter the population of T cell subsets. Parallels with other virus infections give rise to possibility of CD4⁺ T cell death, perhaps with concurrent CD8⁺ stimulation or that the virus may act as the level of T cell intra-thymic differentiation (Drew T.W. (2000). *Veterinary Research* 31, 27-39). Shimizu (Shimizu M., *et al.*, (1996). *Veterinary Immunological Immunopathology* 50, 19-27) proposed that the cause of CD8⁺ cell proliferation induced as a consequence of PRRSV infection. Based on the findings that PRRSV ORF 5 region has an apoptotic effect on transfected cells (Suarez P., *et al.*, (1996). *J. Virology* 70, 2876-2882).

PRRSV may cause immunosuppression in its host. It was uncertain that whether the decrease in CD8⁺ cytotoxic T cells correlated with the apoptotic ability of ORF 5 region of PRRSV. Apoptosis is a critical process in the normal functioning of the immune system. Thus the destructive of the cytotoxic effects of CD8⁺ T cells on target cells are all mediated by apoptosis. The difference of response in T cell population after immunization in mice and pigs showed that there was a variation in pathogenicity of the homologous virus among different hosts.

Pigs like other species have the typical CD4⁺CD8⁺ and CD4⁺CD8⁺ T lymphocytes in their peripheral blood and secondary lymphoid organs. These cells are shown to have helper and cytolytic functions. However, unlike humans and mice, swine also have a prominent CD4/CD8 double positive (DP) lymphocyte population, comprising between 8% and 64% of the circulating pool of small resting T lymphocytes (Zuckennann F.A-(1999). *Veterinary Immunology and Immunopathology* 72, 55-66). The relative proportion and the absolute

number of this lymphocyte subset in the peripheral blood of swine increase gradually with age. This cell population represents an independent cell lineage with no direct relation to mature CD4 or CD8 single positive T cells. However, dual DP cells could represent memory CD4⁺ T helper lymphocytes that have acquired the CD8 antigen upon prior sensitization and retained it after reversion to small lymphocytes. These cells are capable of helping B cells to produce antibody. And because of the predominance of DP cells in older pigs, it is likely that these play a major role in protective immunity due to the co-expression of CD4 and CD8 might be advantageous for recognition of nominal antigen.

This example shows that DNA immunization with a plasmid encoding GP5 of PRRSV is able to develop both the humoral and cell-mediated immunity in mice and its natural host. Therefore, it appears that the ORF 5 may be a good candidate for a recombinant type vaccine against this regional PRRSV. However, the genomic variability of the ORF 5 genes which was recently reported between North American and European strains (Mardassi H., *et al.*, (1995). *Archives of Virology* 140,1405-1418), indicates that these variabilities may be evaluated significance in terms of the antigenic determinants involved in protection for the production of a vaccine of the present invention.

DNA immunization is not sufficient to inhibit virus persistence and shedding in the respiratory tract after virus challenge (Pirzadeh B. and Dea S. (1998). *J. General Virology* 79, 989-999). And it was found that mucosal immunity is believed to play a role in protection against PRRSV infection, virus persistence and shedding, thus this aspect of immunity against PRRSV was evaluated. Accordingly, efforts to develop a multivalent DNA vaccine for PRRS and associated serological tests under another PRRSV antigen, ORF 6, was attempted, however, and a preliminary humoral immune response obtained. Recently, an infectious clone was generated (Meulenberg J.J.M., *et al.*, (1998). *J. Virology*. 72, 380-387) and it is contemplated

that this could be mutagenised at a specific site to introduce a marker or to reduce the virulence and develop a safe and effective marker vaccine for this virus.

EXAMPLE 9

Demonstration of a Bivalent Vaccine against Two Pathogens

The example herein describes construction of bivalent vaccina with efficacy against two pathogens. As would be understood by one of ordinary skill in the art, bi- or multivalent vaccines of the present invention may be created using modern molecular cloning technology in light of the disclosures herein.

Laryngotracheitis Virus (ILTV) is a DNA herpesvirus that causes a severe upper respiratory disease in chicken. Infectious Bronchitis Virus (IBV) is a member of the coronavirus, a single-stranded RNA virus. They are highly species-specific and both viruses cause significant economic loss in the poultry industry if not under control. In one embodiment of the invention, a bivalent virus was created to both diseases. A recombinant vaccina was created by deleting the TK gene from the ILTV genome and inserting the S1 gene subcloned from the IBV genome. Recombinant virus was generated by transforming chicken embryo kidney cells with the recombinant ILTV-IBV construct. Recombinant virus showed to induce immunity against both ITLV and IBV in chickens challenged with ITLV and IBV. It is possible to administer the vaccine in water and/or mist.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically

and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein.

The compounds, methods, procedures and techniques described herein are presently representative of preferred embodiments, are intended to be exemplary and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims. It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

CLAIMS

1. An orally acceptable immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a plant, wherein said immunogen is expressed in the plant at a level such that upon oral administration of said composition to an animal, an immunogenic response is observed.
2. The orally acceptable immunogenic composition of claim 1 wherein said HCV, IBDV, IBV, ILTV or PRRSV immunogen is capable of generating an immunogenic response to HCV, IBDV, IBV, ILTV or PRRSV when the immunogen interacts with a mucosal membrane.
3. The orally acceptable immunogenic composition of claim 1 wherein the HCV, IBDV, IBV, ILTV or PRRSV immunogen is capable of binding a glycosylated molecule on the surface of a membrane of a mucosal cell.
4. The orally acceptable immunogenic composition of claim 1 wherein said HCV, IBDV, IBV, ILTV or PRRSV immunogen is a chimeric protein.
5. An orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein said immunogen is expressed in the plant at a level such that upon oral administration of said composition to an animal, an immunogenic response is observed, said viral immunogen being an immunogenic protein from a cholera virus of swine.
6. An orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein said immunogen is

- expressed in the plant at a level such that upon oral administration of said composition to an animal, an immunogenic response is observed, said viral immunogen being an immunogenic protein from a porcine reproductive and respiratory syndrome virus.
7. An orally acceptable immunogenic composition comprising unpurified or partially purified recombinant immunogen expressed in a plant, wherein said immunogen is expressed in the plant at a level such that upon oral administration of said composition to an animal, an immunogenic response is observed, said viral immunogen being an immunogenic protein from an infectious bursal disease virus.
 8. A vaccine comprising an immunogen of hog cholera virus, porcine reproductive and respiratory syndrome virus or infectious bursal disease virus expressed in a plant, wherein said immunogen is capable of binding a glycosylated molecule on a surface of a membrane mucosal cell.
 9. The vaccine of claim 8, wherein said immunogen is a hog cholera virus immunogen.
 10. The vaccine of claim 8, wherein said immunogen is a porcine reproductive and respiratory syndrome virus immunogen.
 11. The vaccine of claim 8, wherein said immunogen is an infectious bursal disease virus.
 12. A plant composition comprising a viral antigen which triggers production of antibodies and which is derived from a HCV, IBDV, IBV, ILTV or PRRSV antigen and plant material; said antigen being a product produced by the method of expressing said

immunogen in a transgenic plant, said plant material being in a form chosen from the group consisting of a whole plant, plant part, or a crude plant extract.

13. A transgenic plant expressing a nucleotide sequence which encodes a recombinant viral antigenic protein, said recombinant protein derived from a cholera virus of swine, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus.
14. The plant of claim 13 wherein said protein is chimeric.
15. The plant of claim 13 wherein said plant is *Arabidopsis*.
16. A vaccine composition comprising:

a recombinant viral antigenic protein, said protein produced in a plant and derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or infectious bursal disease virus; and plant material, wherein said vaccine composition is capable of eliciting an immune response upon administration to an animal.
17. A food comprising transgenic plant material capable of being ingested for its nutritional value, said transgenic plant expressing a recombinant immunogen derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus.
18. The food of claim 17 wherein said plant is *Arabidopsis*.

19. A food comprising transgenic plant material capable of being ingested for its nutritional value, said transgenic plant expressing a recombinant immunogen derived from hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus.
20. The food of claim 19 wherein said immunogen is derived from hog cholera virus.
21. The food of claim 19 wherein said immunogen is derived from a porcine reproductive and respiratory syndrome virus.
22. The food of claim 19 wherein said immunogen is derived from hog cholera virus.
23. The food of claim 19 wherein said plant is selected from the group consisting of: tomato and potato.
24. The food of claim 19 wherein said transgenic plant material is selected from the group consisting of: edible fruit, leaves, juices, roots, and seed of said plant.
25. A method for constructing a transgenic plant cell comprising: constructing a DNA vector by operably linking a DNA sequence encoding a recombinant viral antigenic protein, said recombinant protein derived from a hog cholera virus, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus to a plant-functional promoter capable of directing the expression of said DNA sequence in said plant; and transforming a plant cell with said DNA vector.

26. The method of claim 25, wherein said transforming comprises *Arabidopsis* mediated transformation.
27. A method for producing a pharmaceutical vaccine composition, wherein the pharmaceutical vaccine composition consists of a recombinant viral antigenic protein, comprising the steps of:
- constructing a DNA vector by operably linking a DNA sequence encoding said recombinant viral antigenic protein, said recombinant protein derived from a swine virus which is the causative agent of cholera, a porcine reproductive and respiratory syndrome virus or an infectious bursal disease virus, to a plant-functional promoter capable of directing the expression of said DNA sequence in a plant;
- transforming a plant with said DNA vector; and
- recovering said pharmaceutical vaccine composition expressed in said plant.
28. The method of claim 27 wherein said plant is an *Arabidopsis* plant.
29. An immunogenic composition comprising unpurified or partially purified recombinant HCV, IBDV, IBV, ILTV or PRRSV immunogen expressed in a bacteria, wherein said immunogen is expressed in the bacteria at a level such that upon administration of said composition to an animal, an immunogenic response is observed.

30. The immunogenic composition of claim 29 wherein said HCV, IBDV, IBV, ILTV or PRRSV immunogen is unpurified from said bacteria.
31. The immunogenic composition of claim 29 wherein the HCV, IBDV, IBV, ILTV or PRRSV immunogen is partly purified from said bacteria.
32. The immunogenic composition of claim 29 wherein said HCV, IBDV, IBV, ILTV or PRRSV immunogen is a chimeric protein.
33. The immunogenic composition of claim 29 wherein said administration comprises injection.
34. The immunogenic composition of claim 29 wherein said administration comprises oral ingestion.
35. A vaccine comprising an immunogen of hog cholera virus, porcine reproductive and respiratory syndrome virus or infectious bursal disease virus expressed in a bacteria.
36. The vaccine of claim 35, wherein said immunogen is a hog cholera virus immunogen.
37. The vaccine of claim 35, wherein said immunogen is a porcine reproductive and respiratory syndrome virus immunogen.
38. The vaccine of claim 36, wherein said immunogen is an infectious bursal disease virus.

39. A method for producing an immunogenic response to an immunogen in an animal, comprising the steps of: expressing a recombinant immunogen in a bacteria, wherein said immunogen is expressed in the bacteria at a level such that upon administration of said bacteria to an animal, an immunogenic response to said immunogen is observed.
40. The method of claim 39, wherein said immunogen is derived from HCV, IBDV, IBV, ILTV or PRRSV.
41. The method of claim 40, wherein said immunogen is partly-purified from said bacteria.

Synthetic Peptide
KDEL (SEQ ID NO: 1)

Synthetic Peptide
HDEL (SEQ ID NO: 2)

DNA Sequences of HCV2.5

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO: 3

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atgaaagccc tattggcatg ggcagtgata gcaatcgcgc tgtaccagcc tgtagcagcc
gagaatataa ctcaatggaa cctgagtgac aacggcacia gcggcatcca gcgagctatg
tatctcagag ggggtcaatag gagcttacat gggatctggc ctgaaaaaat atgcaagggg
gtccccactc atctggccac tgatacggaa ctgacagaga tacgcgggat gatggacgcc
agtgaaggga cgaactatac gtgtttagg ttgcagagac acgaatggaa taaacatgga
tggtgttaact ggtacaacat agacccttgg attcagttaa tgaacaggac ccaagcaaat
ttgacagaag gccctccaaa taaagagtgc gctgtgactt gcaggtatga caacaatacc
gacgttaacg tggttaccca ggccaggaat agaccaacca ctctgactgg ctgcaagaaa
gggaaaaatt tttcatttgc aggtacggtc atagagggcc catgcaattt caacgtatcc
gtggaggaca tcttatatgg agaccatgag tgtggcagcc tgtttcagga caccgctctg
tacctattag atggaatgac caacactata gagaaagcca ggcagggtgc ggcaagagtc
acatcttggc tagggaggca actcagtagc acagggaaga agttggagag aggaagcaaa
acctgggttcg tggcctatgc cctgtcacct tattgcaatg taacaaggaa aatagggtac
atatggtaca cgaacaattg cactccggca tgcctccaa aaaacacaaa aataataggg
ccaggaaaat ttgataccaa cgcagaagac gggaagattc tcatgaaat gggggccac
ctatcagagt tcttgttcgt ttctctggta atcctgtctg acttcgcccc agaaacagcc
agtacgttat acctaatctt aactatgca atccctcagt cctatgaaga acctgagggg
tgccgatatga accagctcaa cctaacgggtg ggacttagga cagaagatgt ggtaccatca
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aagggtggctc tactgtttga agaggtagga cagggtataa agctagctct acgggactg
agggatttaa ctagagtctg gaacagtgca tcaaccactg cattcctcat ctgcttgata
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caagggcggg tgtcctgcaa ggaagactac acctggaaag aatacaacca tggtttgag
gggcccgtag gggctgaagg tctcactacc actgcagggt cctttaaagt tatagcactt
ctggatgacg ggacagtcag ggccatctgc actgcagggt gggctttgcc cacctcagta
aatgtggtca gtaggagata cctggcatca ttacacaaga gggctttgcc cacctcagta
acatttgaac tcctatttga tgggactagt ccagcaatag aggagatggg agatgacttt
ggatttgggc tgtgcccttt tgacacaacc cccgtggtaa aagggaagta caataccact
ttgttaaacg gtagtgcctt ctacctagtc tgcccaatag gatggacggg tgtcatagag
tgacacggcag taagcctcac aaccttgaga acagaagtgg tgaagacctt caagagagag
aagcctttcc cgcacagagt ggattgcgtg accactatag tagaaaaaga agacctgttc
tactgcaagt tgggggtaa ttggacatgt gtgaaaggca acccggtgac ctacatgggg
gggcaagtaa aacaatgcag gtgggtgcgg tttgacttca aggagcccga tgggctccca
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ggcaacacca ccgtcaaggt acacgcgttg gatggaagac tgggccccat gccgtgcaga
cccaaaagaaa ttgtctctag tgggggacct gtaaggaaaa ctctctgcac tttcaattac
acaaagacac taagaaataa gtactatgag cccaaggaca gctatttcca gcaatatatg
cttaaggggc agtaccaata ctgggttgat ctggacgtga ctgaccacca cacagactac
tttgccgaat ttgttgtctt ggtggttagtg gcactattag gggggaggta cgttctgtgg
ctaatagtga cctatatagt tctaacagag caactcgctg ctggtttaca gctaggccaa
ggcgaggcgg tactgatagg gaacttaate acccacacgg acaatgaggt ggtggtatag
ttcttactgc tctacctgat aataagagac gagcccataa agaaatggat actactgctg
tttcatggaa ttctgcagat atccagcaca gtggcgcccg ctcgagtcta g

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DNA Sequences of HCV1.25Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:4

ATG CGG CTA GCC TGC AAG GAA GAT TAC AGG TAT GCG ATA TCA TCA ACC AAT GAG ATA GGG
 CCG CTA GGG GCT GAA GGT CTC ACT ACC ACC TGG AAA GAA TAC AAC CAT GGT TTG CAG CTG
 GAT GAC GGG ACA GTC AGG GCC ATC TGC ACT GCA GGG TCC TTT AAA GTT ATA GCA CTT AAT
 GTG GTC AGT AGG AGA TAC CTG GCA TCA TTA CAC AAG AGG GCT TTG CCC ACC TCA GTA ACA
 TTT GAA CTC CTA TTT GAT GGG ACT AGT CCA GCA ATA GAG GAG ATG GGA GAT GAC TTT GGA
 TTT GGG CTG TGC CCT TTT GAC ACA ACC CCC GTG GTA AAA GGG AAG TAC AAT ACC ACT TTG
 TTA AAC GGT AGT GCT TTC TAC CTA GTC TGC CCA ATA GGA TGG ACG GGT GTC ATA GAG TGC
 ACG GCA GTA AGC CTC ACA ACC TTG AGA ACA GAA GTG GTG AAG ACC TTC AAG AGA GAG AAG
 CCT TTC CCG CAC AGA GTG GAT TGC GTG ACC ACT ATA GTA GAA AAA GAA GAC CTG TTC TAC
 TGC AAG TTG GGG GGT AAT TGG ACA TGT GTG AAA GGC AAC CCG GTG ACC TAC ATG GGG GGG
 CAA GTA AAA CAA TGC AGG TGG TGC GGT TTT GAC TTC AAG GAG CCC GAT GGG CTC CCA CAC
 TAC CCC ATA GGC AAG TGC ATC CTA GCA AAT GAG ACG GGT TAC AGG GTA GTG GAT TCC ACA
 GAC TGC AAC AGA GAT GGC GTC GTT ATC AGC ACT GAA GGA GAA CAC GAG TGC TTG ATT GGC
 AAC ACC ACC GTC AAG GTA CAC GCG TTG GAT GGA AGA CTG GGC CCC ATG CCG TGC AGA CCC
 AAA GAA ATT GTC TCT AGT GCG GGA CCT GTA AGG AAA ACT TCC TGC ACT TTC AAT TAC ACA
 AAG ACA CTA AGA AAT AAG TAC TAT GAG CCC AAG GAC AGC TAT TTC CAG CAA TAT ATG CTT
 AAG GGC GAG TAC CAA TAC TGG TTT GAT CTG GAC GTG ACT GAC CAC CAC ACA GAC TAC TTT
 GCC GAA TTT GTT GTC TTG GTG GTA GTG GCA CTA TTA GGG GGG AGG TAC GTT CTG TGG CTA
 ATA GTG ACC TAT ATA GTT CTA ACA GAG CAA CTC GCT GCT GGT TTA CAG CTA GGC CAA GGC
 GAG GCG GTA CTG ATA GGG AAC TTA ATC ACC CAC ACG GAC AAT GAG GTG GTG GTA TAC TTC
 TTA CTG CTC TAC CTG ATA ATA AGA GAC GAG CCC ATA AAG AAA TGG ATA CTA CTG CTG TTT
 CAT GGA ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT CGA GTC TAG

DNA Sequences of HCV1.25Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:5

ATG CGG CTA GCC TGC AAG GAA GAT TAC AGG TAT GCG ATA TCA TCA ACC AAT GAG ATA GGG
CCG CTA GGG GCT GAA GGT CTC ACT ACC ACC TGG AAA GAA TAC AAC CAT GGT TTG CAG CTG
GAT GAC GGG ACA GTC AGG GCC ATC TGC ACT GCA GGG TCC TTT AAA GTT ATA GCA CTT AAT
GTG GTC AGT AGG AGA TAC CTG GCA TCA TTA CAC AAG AGG GCT TTG CCC ACC TCA GTA ACA
TTT GAA CTC CTA TTT GAT GGG ACT AGT CCA GCA ATA GAG GAG ATG GGA GAT GAC TTT GGA
TTT GGG CTG TGC CCT TTT GAC ACA ACC CCC GTG GTA AAA GGG AAG TAC AAT ACC ACT TTG
TTA AAC GGT AGT GCT TTC TAC CTA GTC TGC CCA ATA GGA TGG ACG GGT GTC ATA GAG TGC
ACG GCA GTA AGC CTC ACA ACC TTG AGA ACA GAA GTG GTG AAG ACC TTC AAG AGA GAG AAG
CCT TTC CCG CAC AGA GTG GAT TGC GTG ACC ACT ATA GTA GAA AAA GAA GAC CTG TTC TAC
TGC AAG TTG GGG GGT AAT TGG ACA TGT GTG AAA GGC AAC CCG GTG ACC TAC ATG GGG GGG
CAA GTA AAA CAA TGC AGG TGG TGC GGT TTT GAC TTC AAG GAG CCC GAT GGG CTC CCA CAC
TAC CCC ATA GGC AAG TGC ATC CTA GCA AAT GAG ACG GGT TAC AGG GTA GTG GAT TCC ACA
GAC TGC AAC AGA GAT GGC GTC GTT ATC AGC ACT GAA GGA GAA CAC GAG TGC TTG ATT GGC
AAC ACC ACC GTC AAG GTA CAC GCG TTG GAT GGA AGA CTG GGC CCC ATG CCG TGC AGA CCC
AAA GAA ATT GTC TCT AGT GCG GGA CCT GTA AGG AAA ACT TCC TGC ACT TTC AAT TAC ACA
AAG ACA CTA AGA AAT AAG TAC TAT GAG CCC AAG GAC AGC TAT TTC CAG CAA TAT ATG CTT
AAG GGC GAG TAC CAA TAC TGG TTT GAT CTG GAC GTG ACT GAC CAC CAC ACA GAC TAC TTT
GCC GAA TTT GTT GTC TTG GTG GTA GTG GCA CTA TTA GGG GGG AGG TAC GTT CTG TGG CTA
ATA GTG ACC TAT ATA GTT CTA ACA GAG CAA CTC GCT GCT GGT TTA CAG CTA GGC CAA GGC
GAG GCG GTA CTG ATA GGG AAC TTA ATC ACC CAC ACG GAC AAT GAG GTG GTG GTA TAC TTC
TTA CTG CTC TAC CTG ATA ATA AGA GAC GAG CCC ATA AAG AAA TGG ATA CTA CTG CTG TTT
CAT TAG

DNA Sequences of HCV1.25Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:6

ATG CGG CTT GCC TGT AAG GAA GAT TAC AGG TAT GCG ATA TCA TCA ACC AAT GAG ATA GGG
CCG CTA GGG GCT GAA GGT CTC ACT ACC ACC TGG AAA GAA TAC AAC CAT GGT TTG CAG CTG
GAT GAC GGG ACA GTC AGG GCC ATC TGC ACT GCA GGG TCC TTT AAA GTT ATA GCA CTT AAT
GTG GTC AGT AGG AGA TAC CTG GCA TCA TTA CAC AAG AGG GCT TTG CCC ACC TCA GTA ACA

TTT GAA CTC CTA TTT GAT GGG ACT AGT CCA GCA ATA GAG GAG ATG GGA GAT GAC TTT GGA
TTT GGA CTT TGT CCT TTT GAC ACA ACC CCA GTG GTT AAA GGT AAG TAC AAT ACC ACT TTG
TTA AAC GGT AGT GCT TTC TAC CTA GTC TGC CCA ATA GGA TGG ACG GGT GTC ATA GAG TGC
ACG GCA GTA AGC CTC ACA ACC TTG AGA ACA GAA GTG GTG AAG ACC TTC AAG AGA GAG AAG
CCT TTC CCG CAC AGA GTG GAT TGC GTG ACC ACT ATA GTA GAA AAA GAA GAC CTG TTC TAC
TGC AAG TTG GGG GGT AAT TGG ACA TGT GTG AAA GGC AAC CCG GTG ACC TAC ATG GGT GGA
CAA GTT AAA CAA TGT AGG TGG TGT GGT TTT GAC TTC AAG GAG CCC GAT TGG CTC TCA CAC
TAC CCT ATT GGT AAG TGC ATC CTA GCA AAT GAG ACG GGT TAC AGG GTA GTG GAT TCC ACA
GAC TGC AAC AGA GAT GGC GTC GTT ATC AGC ACT GAA GGA GAA CAC GAG TGC TTG ATT GGC
AAC ACC ACC GTC AAG GTA CAC GCG TTG GAT GGA AGA CTT GGT CCA ATG CCG TGT AGA CCG
AAA GAA ATT GTC TCT AGT GCG GGA CCT GTA AGG AAA ACT TCC TGC ACT TTC AAT TAC ACA
AAG ACA CTA AGA AAT AAG TAC TAT GAG CCC AAG GAC AGC TAT TTC CAG CAA TAT ATG CTT
AAG GGC GAG TAC CAA TAC TGG TTT GAT CTG GAC GTG ACT GAC CAC CAC ACA GAC TAC TTT
GCC GAA TTT GTT GTC TTG GTG GTT GTG GCA CTT TTA GGA GGT AGG TAC GTT CTT TGG CTA
ATA GTG ACC TAT ATA GTT CTA ACA GAG CAA CTC GCT GGT TTA CAG CTA GGC CAA GGC
GAG GCG GTA CTG ATA GGG AAC TTA ATC ACC CAC ACG GAC AAT GAG GTG GTG GTA TAC TTC
TTA CTG CTC TAC CTG ATA ATA AGA GAC GAG CCC ATA AAG AAA TGG ATT CTT CTT CTC TTT
CAT TAG

Species: *Arabidopsis thaliana*

Synthetic oligonucleotide

SEQ ID NO:7

ATCTTCCATGTCGCAACTCC

Species: *Arabidopsis thaliana*

Synthetic oligonucleotide

SEQ ID NO:8

CAAATGTAGCGACCAGAAGC

DNA Sequences of HCV1.25

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:9

GTAGGATCCATGCGGCTTGCCTGTAAGG

DNA Sequences of HCV

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:10

AAAACCACACCACCTACATTGTTTAACTTGTCCACCCATG

DNA Sequences of HCV

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:11

ATAGGATCCATGAAAGCCCTATTGGCATGGGCAGTGATG

DNA Sequences of HCV

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:12

CCCGGGAATTCCATGAAACAGCAGTAGTATCCATTCTT

DNA Sequences of HCV

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:13

ATTGGATCCATGCGGCTAGCCTGCAAGGAAGATTAC

DNA Sequences of HCV

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:14

GACAGCATCATTACACAAGAGG

DNA Sequences of HCVSpecies: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:15

GTACTTACCTTTAACCACTGGGGTTGTGTCAAAAGGACAAAGTCCAAA

DNA Sequences of HCVSpecies: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:16

TCTTTCGGTCTACACGGCATTGGACCAAGTCTT

DNA SEQUENCES 17-28SPECIES: genus *Arterivirus* of *Arteriviridae*

GTATGAACTTGCAGGATG (SEQ ID NO:17)

GCCGACAATACCATGTGCTG (SEQ ID NO:18)

GGCGCAGTGACTAAGAGA (SEQ ID NO:19)

GTAAGTGAACACCATATGCTG (SEQ ID NO:20)

CCTCCTGTATGAACTTGC (SEQ ID NO:21)

AGGTCCTCGAACTTGAGCTG (SEQ ID NO:22)

GGCCTGTCGT CCGGCGACCC GATCACCTCT GTGTCTAACA CCATTTACAG ,
TTTGGTGATC TACGCACAG CATATGGTGT TCAGTTACAT (SEQ ID NO:23)GGCCTGTCGT CTGGCGACCC GATCACCTCT GTGTCTAACA CCATTTATAG
TTTGGTGATA TATGCACAG CATATGGTGT TCAGTTACA (SEQ ID NO:24)GGCCTGTCGT CCGGCGACCC GATCAGCTCT GTGTCTAACA CCATTTACAG
TTTGGTGATC TACGCACAG CATATGGTGT TCAGTTACAT (SEQ ID NO:25)GTTTTTCAT AAAAAAAGGT TAGAAAATAA AAATAAAATC TCCATAGCTG
GTGGTTATTT TAAAAGTTAG AATGAGACTA TAAGCAATGG (SEQ ID NO:26)

GGCCTGTCGT CCGGCGACCC GATCACCTCT GTGTCTAACA CCATTACAG
TTTGGTGATC TACGCACAG CATATGGTGT TCAGTTACAT (SEQ ID NO:27)

GGCCTGTCGT CTGGCGACCC GATCACCTCT GTGTCTAACA CCATTACAG
TTTGGTGATC TACGCACAG CATATGGTGC TCAGTTACTT (SEQ ID NO:28)

DNA SEQUENCES 29-34

chicken Infectious Bronchitis Virus (IBV)

coronaviridae family, genus *coronavirus*

CCGAATTCGCTATGAAAACCTGAACAAAA (SEQ ID NO:29)

GGGTCGACATCCATAACTAACACAAGGG (SEQ ID NO:30)

TCAAAGCTTCANGGNGCNTA (SEQ ID NO:31)

CTCGAATTCNGTRTAYTGRCA (SEQ ID NO:32)

GTATTCTGCTTTAAAAAG (SEQ ID NO:33)

AGCTCACC ACTATAAACA (SEQ ID NO:34)

PROTEIN SEQUENCES 35-52

chicken Infectious Bronchitis Virus (IBV)

coronaviridae family, genus *coronavirus*

HGGAY (SEQ ID NO:35)

SENNAGTAPSC TAG (SEQ ID NO:36)

KSGSNSCPLTGLIPKGQIRISAMRSVNSRLHI (SEQ ID NO:37)

CQYNTG (SEQ ID NO:38)

TISYNAGTACTAG (SEQ ID NO:39)

KAGSNGLIPKGQIRISAMRSVNSRLHL (SEQ ID NO:40)

SENNAGTAPSC TAG (SEQ ID NO:41)

KSGSNSCPLTGLIPKGQIRISAMRSVNSRLHF (SEQ ID NO:42)

SENNAGAPSCCTAG (SEQ ID NO:43)

KSGSNSCPLTGLIPKGQIRIRMASVNSRLTI (SEQ ID NO:44)

SENNAGTAPSCCTAG (SEQ ID NO:45)

KAGSNSCPLTGLIPKGQIRISAMRSVNSRLHI (SEQ ID NO:46)

SENNAPTQYSCTAG (SEQ ID NO:47)

KSGSNCPLTGLIPKGQIRISAMRSVNSRLF (SEQ ID NO:48)

TISYTAPSCCTAG (SEQ ID NO:49)

ISGSNSCPLTGLIPRISAMRNVNSRLLI (SEQ ID NO:50)

SEINAGSCTAG (SEQ ID NO:51)

KAGSNSCLIPKGQIRISAMRSVNSRLHA (SEQ ID NO:52)

DNA Sequences of HCV1.25

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO:53

ATG CGG CTA GCC TGC AAG GAA GAT TAC AGG TAT GCG ATA TCA TCA ACC AAT GAG ATA GGG
CCG CTA GGG GCT GAA GGT CTC ACT ACC ACC TGG AAA GAA TAC AAC CAT GGT TTG CAG CTG
GAT GAC GGG ACA GTC AGG GCC ATC TGC ACT GCA GGG TCC TTT AAA GTT ATA GCA CTT AAT
GTG GTC AGT AGG AGA TAC CTG GCA TCA TTA CAC AAG AGG GCT TTG CCC ACC TCA GTA ACA
TTT GAA CTC CTA TTT GAT GGG ACT AGT CCA GCA ATA GAG GAG ATG GGA GAT GAC TTT GGA
TTT AGG CTG TGC CCT TTT GAC ACA ACC CCC GTG GTA AAA GGG AAG TAC AAT ACC ACT TTG
TTA AAC GGT AGT GCT TTC TAC CTA GTC TGC CCA ATA GGA TGG ACG GGT GTC ATA GAG TGC
ACG GCA GTA AGC CTC ACA ACC TTG AGA ACA GAA GTG GTG AAG ACC TTC AAG AGA GAG AAG
CCT TTC CCG CAC AGA GTG GAT TGC GTG ACC ACT ATA GTA GAA AAA GAA GAC CTG TTC TAC
TGC AAG TTG GGG GGT AAT TGG ACA TGT GTG AAA GGC AAC CCG GTG ACC TAC ATG GGG GGA
CAA GTA AAA CAA TGC AGG TGG TGC GGT TTT GAC TTC AAG GAG CCC GAT GGG CTC CCA CAC
TAC CCC ATA GGC AAG TGC ATC CTA GCA AAT GAG ACG GGT TAC AGG GTA GTG GAT TCC ACA
GAC TGC AAC AGA GAT GGC GTC GTT ATC AGC ACT GAA GGA GAA CAC GAG TGC TTG ATT GGC
AAC ACC ACC GTC AAG AGA GTA CAC GCG TTG GAT GGA AGA CTG GGC CCC ATG CCG TGC AGA
CCC
AAA GAA ATT GTC TCT AGT GCG GGA CCT GTA AGG AAA ACT TCC TGC ACT TTC AAT TAC ACA

AAG ACA CTA AGA AAT AAG TAC TAT GAG CCC AAG GAC AGC TAT TTC CAG CAA TAT ATG CTT
AAG GGC GAG TAC CAA TAC TGG TTT GAT CTG GAC GTG ACT GAC CAC CAC ACA GAC TAC TTT
GCC GAA TTT GTT GTC TTG GTG GTA GTG GCA CTA TTA GGG GGG AGG TAC GTT CTG TGG CTA
ATA GTG ACC TAT ATA GTT CTA ACA GAG CAA CTC GCT GCT GGT TTA CAG CTA GGC CAA GGC
GAG GCG GTA CTG ATA GGG AAC TTA ATC ACC CAC ACG GAC AAT GAG GTG GTG GTA TAC TTC
TTA CTG CTC TAC CTG ATA ATA AGA GAC GAG CCC ATA AAG AAA TGG ATA CTA CTG CTG TTT
CAT GGA ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT CGA GTC TAG

DNA Sequences of MHCV1.25

Species: *Pestivirus* genus of the *Flaviviridae*

SEQ ID NO: 54

ATG CGG CTT GCC TGT AAG GAA GAT TAC AGG TAT GCG ATA TCA TCA ACC AAT GAG ATA GGG
CCG CTA GGG GCT GAA GGT CTC ACT ACC ACC TGG AAA GAA TAC AAC CAT GGT TTG CAG CTG
GAT GAC GGG ACA GTC AGG GCC ATC TGC ACT GCA GGG TCC TTT AAA GTT ATA GCA CTT AAT
GTG GTC AGT AGG AGA TAC CTG GCA TCA TTA CAC AAG AGG GCT TTG CCC ACC TCA GTA ACA
TTT GAA CTC CTA TTT GAT GGG ACT AGT CCA GCA ATA GAG GAG ATG GGA GAT GAC TTT GGA
TTT GGA CTT TGT CCT TTT GAC ACA ACC CCA GTG GTA AAA GGT AAG TAC AAT ACC ACT TTG
TTA AAC GGT AGT GCT TTC TAC CTA GTC TGC CCA ATA GGA TGG ACG GGT GTC ATA GAG TGC
ACG GCA GTA AGC CTC ACA ACC TTG AGA ACA GAA GTG GTG AAG ACC TTC AAG AGA GAG AAG
CCT TTC CCG CAC AGA GTG GAT TGC GTG ACC ACT ATA GTA GAA AAA GAA GAC CTG TTC TAC
TGC AAG TTG GGG GGT AAT TGG ACA TGT GTG AAA GGC AAC CCG GTG ACC TAC ATG GGT GGA
CAA GTT AAA CAA TGT AGG TGG TGT GGT TTT GAC TTC AAG GAG CCT GAT GGT CTC CCA CAT
TAC CCT ATT GGT AAG TGC ATC CTA GCA AAT GAG ACG GGT TAC AGG GTA GTG GAT TCC ACA
GAC TGC AAC AGA GAT GGC GTC GTT ATC AGC ACT GAA GGA GAA CAC GAG TGC TTG ATT GGC
AAC ACC ACC GTC AAG AGA GTA CAC GCG TTG GAT GGA AGA CTT GGT CCA ATG CCG TGT AGA
CCG

AAA GAA ATT GTC TCT AGT GCG GGA CCT GTA AGG AAA ACT TCC TGC ACT TTC AAT TAC ACA
AAG ACA CTA AGA AAT AAG TAC TAT GAG CCC AAG GAC AGC TAT TTC CAG CAA TAT ATG CTT
AAG GGC GAG TAC CAA TAC TGG TTT GAT CTG GAC GTG ACT GAC CAC CAC ACA GAC TAC TTT
GCC GAA TTT GTT GTC TTG GTG GTT GTG GCA CTT TTA GGA GGT AGG TAC GTT CTT TGG CTA
ATA GTG ACC TAT ATA GTT CTA ACA GAG CAA CTC GCT GCT GGT TTA CAG CTA GGC CAA GGC
GAG GCG GTA CTG ATA GGG AAC TTA ATC ACC CAC ACG GAC AAT GAG GTG GTG GTA TAC TTC
TTA CTG CTC TAC CTG ATA ATA AGA GAC GAG CCC ATA AAG AAA TGG ATT CTT CTT CTC TTT
CAT GGA ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT CGA GTC TAG

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NOVEL VACCINE COMPOSITIONS AND METHODS OF VACCINE PREPARATION FOR VETERINARY AND HUMAN DISEASES

(57) Abstract: The anti-pathogen vaccine of the present invention is produced in recombinant bacteria and/or transgenic plants and then administered through standard vaccine introduction method or through standard vaccine introduction method or through the oral administration. A DNA sequence encoding for the expression of an antigen of a pathogen is isolated and ligated to a promoter which can regulate the production of the surface antigen in a bacterial or transgenic plant. Preferably, a foreign gene is expressed in a portion of the plant or bacteria, and all or part of the antigen expressing plant or bacteria used for vaccine administration. In a preferred procedure, the vaccine is administered through the consumption of the edible plant as food, or the bacteria administered orally.



WO 2003/013598 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/25126

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/187 A61K39/215 A61K39/12 C12N15/82 C07K14/165
C07K14/18 C07K14/03 C07K14/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM HYUNSOON ET AL: "Expression of hog cholera virus in transgenic potato plants." PLANT BIOLOGY (ROCKVILLE), vol. 2001, 2001, page 142, XP001161300 Joint Annual Meetings of the American Society of Plant Biologists and the Canadian Society of Plant Physiologists; Providence, Rhode Island, USA; July 21-25, 2001 the whole document	1-3,5,8, 9,12,13, 16,17, 19,20, 23-27
Y	----- -/--	4,14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

4 August 2003

Date of mailing of the international search report

10. 11. 2003

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Holtorf, S.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/25126

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>VAN GENNIP A H G P ET AL: "Chimeric classical swine fever viruses containing envelope protein E or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 4-5, 15 October 2000 (2000-10-15), pages 447-459, XP004218115 ISSN: 0264-410X the whole document</p>	4,14
A	<p>WO 99 08706 A (HAMMOND JEFFREY MICHAEL ;JOHNSON MICHAEL ANTHONY (AU); COMMW SCIEN) 25 February 1999 (1999-02-25) page 17 - page 18</p>	
A	<p>HULST M M ET AL: "Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera" JOURNAL OF VIROLOGY, NEW YORK, US, US, vol. 67, no. 9, 1 September 1993 (1993-09-01), pages 5435-5442, XP002080070 ISSN: 0022-538X the whole document</p>	
A	<p>VAN RIJN P A ET AL: "An experimental marker vaccine and accompanying serological diagnostic test both based on envelope glycoprotein E2 of classical swine fever virus (CSFV)" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 17, no. 5, February 1999 (1999-02), pages 433-440, XP004153825 ISSN: 0264-410X the whole document</p>	
A	<p>WIGDOROVITZ A ET AL: "Induction of a Protective Antibody Response to Foot and Mouth Disease Virus in Mice Following Oral or Parenteral Immunization with Alfalfa Transgenic Plants Expressing the Viral Structural Protein VP1" VIROLOGY, ACADEMIC PRESS,ORLANDO, US, vol. 255, no. 2, 15 March 1999 (1999-03-15), pages 347-353, XP004440022 ISSN: 0042-6822 the whole document</p>	
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/25126

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>STREATFIELD S J ET AL: "Plant-based vaccines: unique advantages" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 17-19, 21 March 2001 (2001-03-21), pages 2742-2748, XP004231106 ISSN: 0264-410X page 2745, right-hand column -----</p>	
A	<p>TUBOLY T ET AL: "Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 18, no. 19, April 2000 (2000-04), pages 2023-2028, XP004202498 ISSN: 0264-410X the whole document -----</p>	
A	<p>PLANA DURAN ET AL: "Baculovirus expression of proteins of Reproductive and Respiratory Syndrome Virus strain Olot/91. Involvement of ORF3 and ORF5 proteins in protection" VIRUS GENES, KLUWER ACADEMIC PUBLISHERS, BOSTON, US, vol. 14, no. 1, 1997, pages 19-29, XP002103647 ISSN: 0920-8569 -----</p>	
A	<p>LAFAYETTE PETER R ET AL: "Engineering soybeans for the production of edible vaccines for poultry." PLANT BIOLOGY (ROCKVILLE), vol. 2000, 2000, page 138, XP002250043 Annual Meeting of the American Society of Plant Physiologists; San Diego, California, USA; July 15-19, 2000 the whole document -----</p>	
A	<p>WO 94 20135 A (EDIBLE VACCINES INC ; LAM DOMINIC MAN KIT (US); ARNTZEN CHARLES JOE) 15 September 1994 (1994-09-15) the whole document -----</p>	
A	<p>DANIELL H ET AL: "MEDICAL MOLECULAR FARMING: PRODUCTION OF ANTIBODIES, BIOPHARMACEUTICALS AND EDIBLE VACCINES IN PLANTS" TRENDS IN PLANT SCIENCE, ELSEVIER SCIENCE, OXFORD, GB, vol. 6, no. 5, May 2001 (2001-05), pages 219-226, XP001037864 ISSN: 1360-1385 -----</p>	
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/25126

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHAN CAROL W M ET AL: "Expression of Hog Cholera Virus E2 in transgenic Arabidopsis." PLANT BIOLOGY (ROCKVILLE), vol. 2002, 2002, page 57, XP002250044 Annual Meeting of the American Society of Plant Biologists on Plant Biology; Denver, CO, USA; August 03-07, 2002, 2002 the whole document	12,13, 15, 17-20, 24-26
P,X	----- EP 1 293 573 A (MALTAGEN FORSCHUNG GMBH) 19 March 2003 (2003-03-19) the whole document -----	1-3,5,8, 9,12,13, 16,17, 19,20, 24-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 02/25126

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claim 19 is identical to claim 17. Moreover, claim 22 is referring to the hog cholera virus; said claim should rather refer to the IBDV virus and was searched accordingly. Claim 26 was searched with respect to "Agrobacterium"-mediated transformation and not Arabidopsis-mediated
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see FURTHER INFORMATIN sheet, invention 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: -

Claim 19 is identical to claim 17. Moreover, claim 22 is referring to the hog cholera virus; said claim should rather refer to the IBDV virus and was searched accordingly. Claim 26 was searched with respect to "Agrobacterium"-mediated transformation and not Arabidopsis-mediated transformation.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4,8,12,13,14,15,16-19,23-28 partially, 5,9,20, completely

Orally acceptable immunogenic composition comprising recombinant HCV expressed in a plant wherein said immunogen is highly expressed to confer oral immunisation by interaction with the mucosal membrane; a vaccine comprising said immunogen, a plant composition comprising said viral immunogen, a transgenic expressing said immunogen, a vaccine composition comprising said immunogen, a food comprising said transgenic plant material, a method for constructing a transgenic plant cell by constructing a vector comprising said immunogen specific DNA sequence and transforming said plant cell with said vector, a method for producing a pharmaceutical vaccine composition comprising the steps of constructing a DNA vector, transforming a plant with said vector and recovering said vaccine composition from said plant.

2. claims: 1-4,8,12,13,14,15,16-19,23-28 partially, 7,11,22 completely

Orally acceptable immunogenic composition comprising recombinant IBDV expressed in a plant wherein said immunogen is highly expressed to confer oral immunisation by interaction with the mucosal membrane; a vaccine comprising said immunogen, a plant composition comprising said viral immunogen, a transgenic expressing said immunogen, a vaccine composition comprising said immunogen, a food comprising said transgenic plant material, a method for constructing a transgenic plant cell by constructing a vector comprising said immunogen specific DNA sequence and transforming said plant cell with said vector, a method for producing a pharmaceutical vaccine composition comprising the steps of constructing a DNA vector, transforming a plant with said vector and recovering said vaccine composition from said plant.

3. claims: 1-4,12 partially,

Orally acceptable immunogenic composition comprising recombinant IBV expressed in a plant wherein said immunogen is highly expressed to confer oral immunisation by interaction with the mucosal membrane; a plant composition comprising said viral immunogen.

4. claims: 1-4,12 partially,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Orally acceptable immunogenic composition comprising recombinant ILTV expressed in a plant wherein said immunogen is highly expressed to confer oral immunisation by interaction with the mucosal membrane; a plant composition comprising said viral immunogen.

5. claims: 1-4,8,12,13,14,15,16-19,23-28 partially, 6,10,21 completely

Orally acceptable immunogenic composition comprising recombinant PRRSV expressed in a plant wherein said immunogen is highly expressed to confer oral immunisation by interaction with the mucosal membrane; a vaccine comprising said immunogen, a plant composition comprising said viral immunogen, a transgenic expressing said immunogen, a vaccine composition comprising said immunogen, a food comprising said transgenic plant material, a method for constructing a transgenic plant cell by constructing a vector comprising said immunogen specific DNA sequence and transforming said plant cell with said vector, a method for producing a pharmaceutical vaccine composition comprising the steps of constructing a DNA vector, transforming a plant with said vector and recovering said vaccine composition from said plant.

6. claims: 29-34,35,39-41 partially , 36 completely

Immunogenic composition comprising recombinant HCV immunogen expressed in bacteria, a vaccine comprising an immunogen of HCV expressed in bacteria, a method for producing an immunogenic response to an immunogen comprising the steps of expressing the recombinant immunogen in bacteria at a level such that upon administration to an animal, an immunogenic response is observed.

7. claims: 29-34,35,39-41 partially , 38 completely

Immunogenic composition comprising recombinant IBDV immunogen expressed in bacteria, a vaccine comprising an immunogen of HCV expressed in bacteria, a method for producing an immunogenic response to an immunogen comprising the steps of expressing the recombinant immunogen in bacteria at a level such that upon administration to an animal, an immunogenic response is observed.

8. claims: 29-34,39-41 partially , completely

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Immunogenic composition comprising recombinant IBV immunogen expressed in bacteria, a method for producing an immunogenic response to an immunogen comprising the steps of expressing the recombinant immunogen in bacteria at a level such that upon administration to an animal, an immunogenic response is observed.

9. claims: 29-34,39-41 partially , completely

Immunogenic composition comprising recombinant ILTV immunogen expressed in bacteria, a method for producing an immunogenic response to an immunogen comprising the steps of expressing the recombinant immunogen in bacteria at a level such that upon administration to an animal, an immunogenic response is observed.

10. claims: 29-34,35,39-41 partially , 37completely

Immunogenic composition comprising recombinant PRRSV immunogen expressed in bacteria, a vaccine comprising an immunogen of HCV expressed in bacteria, a method for producing an immunogenic response to an immunogen comprising the steps of expressing the recombinant immunogen in bacteria at a level such that upon administration to an animal, an immunogenic response is observed.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/25126

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9908706	A	25-02-1999	AU 757683 B2	27-02-2003
			AU 8720998 A	08-03-1999
			WO 9908706 A1	25-02-1999
			BR 9811184 A	26-12-2001
			CN 1275084 T	29-11-2000
			EP 1007088 A1	14-06-2000
			JP 2001514871 T	18-09-2001
			NZ 503039 A	27-09-2002
WO 9420135	A	15-09-1994	US 5612487 A	18-03-1997
			AU 6813394 A	26-09-1994
			EP 0728014 A1	28-08-1996
			WO 9420135 A1	15-09-1994
			US 6136320 A	24-10-2000
			US 5914123 A	22-06-1999
			US 6034298 A	07-03-2000
			US 2001053367 A1	20-12-2001
			US 2003138456 A1	24-07-2003
			US 2002006411 A1	17-01-2002
EP 1293573	A	19-03-2003	DE 10145969 A1	10-04-2003
			CN 1422665 A	11-06-2003
			EP 1293573 A2	19-03-2003
			US 2003077640 A1	24-04-2003